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THE ESTIMATION OF AMINO ACID NITROGEN IN ANIMAL TISSUES.

By JAMES MURRAY LUCK.

(From the Department of Chemistry, Stanford University, California.)

(Received for publication, December 29, 1927.)

Of the existing methods for the determination of amino acid nitrogen in animal tissues, those of Folin and Denis (1) and of

CORRECTIONS.

On pages 721 and 722, Vol. lxxiii, No. 2, June, 1927, Table IX, in the heading of the next to the last column, read *0.01 N HCl* for *1.025 N HCl*.

Procedure.

A portion of tissue, approximately 4 to 5 gm. in weight, is excised and dropped into a mortar containing liquid air. The sample is finely powdered, meanwhile being kept thoroughly frozen. A 3 gm. portion of the powder is then weighed out to the nearest mg. The use of a small weighing bottle and a chainomatic balance permits this to be done rapidly and reduces to a negligible amount the error from water condensation and air currents. The material is then transferred quantitatively with boiling 0.01 N acetic acid to a tube 8 × 1 inches, graduated to 50 cc. 30 to 35 cc. of the acetic acid are used in several portions of 5 to 10 cc. each. The tube is promptly immersed in a water bath and left for 7 minutes after

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THE ESTIMATION OF AMINO ACID NITROGEN IN ANIMAL TISSUES.

By JAMES MURRAY LUCK.

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Of the existing methods for the determination of amino acid nitrogen in animal tissues, those of Folin and Denis (1) and of Van Slyke (2) are best known and most extensively employed. Both of them are long and tedious and call for rather large tissue samples. Also methyl alcohol containing zinc chloride is employed as an extractive by Folin and Denis and ethyl alcohol as a protein precipitant by Van Slyke. In both cases there are grounds for believing (1, 3–6) that some of the amino acids are lost in the treatment with alcohol. An attempt has therefore been made to develop a method which would be free from these disadvantages.

The one to be described is a modification of the Greenwald-Bock-Blau (5–7) technique for the determination of the amino acid content of blood. It has been used by the author primarily for the analysis of liver and muscle.

Procedure.

A portion of tissue, approximately 4 to 5 gm. in weight, is excised and dropped into a mortar containing liquid air. The sample is finely powdered, meanwhile being kept thoroughly frozen. A 3 gm. portion of the powder is then weighed out to the nearest mg. The use of a small weighing bottle and a chainomatic balance permits this to be done rapidly and reduces to a negligible amount the error from water condensation and air currents. The material is then transferred quantitatively with boiling 0.01 N acetic acid to a tube 8 × 1 inches, graduated to 50 cc. 30 to 35 cc. of the acetic acid are used in several portions of 5 to 10 cc. each. The tube is promptly immersed in a water bath and left for 7 minutes after

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the temperature of the tissue suspension has reached the boiling point. The tube should be shaken from time to time. After 7 minutes boiling the tube is removed from the bath and allowed to cool. 3 cc. of 50 per cent trichloroacetic acid are now added and the volume accurately made up to 50 cc. with water. The suspension is mixed thoroughly and permitted to stand for 30 minutes. At the end of this time 2 gm. of infusorial earth are added. The mixture is shaken vigorously for 10 to 15 seconds and filtered. Of the filtrate (37 to 38 cc. are quickly obtained) 35 cc. are measured into a 100 cc. beaker and evaporated over a free flame or on a hot plate to about 10 cc. 10 per cent sodium hydroxide is now added until the solution is alkaline to phenolphthalein. Usually 10 to 20 drops are required. Boiling of the alkaline solution is continued for 2 minutes. It is then acidified by adding an excess of glacial acetic acid and the concentration continued with care to a final volume of about 1 cc. The residue is washed into the Van Slyke apparatus for determination of the amino acid nitrogen.

DISCUSSION.

Preparation of the Sample.

The use of liquid air presents several obvious advantages. In the first place the temperature of the sample may be lowered rapidly to a point where postmortem changes proceed immeasurably slowly. That this is an important consideration may be inferred from recent work on the inorganic phosphorus of muscle (8) and from the many investigations of lactic acid metabolism. Of equal importance is the destruction of the cells by freezing and thawing and the obtaining of a brittle mass which may be reduced readily to a very fine powder.

In most work on tissue analysis unfrozen material has been employed—being minced, finely ground with sand or powdered glass, or desiccated at 70–80° (9).

Treatment with 0.01 N Acetic Acid.

The purpose of this part of the procedure is to extract the amino acids, to remove most of the proteins by heat coagulation, and to destroy as rapidly as possible the tissue enzymes.

We have found by trial that the concentration of the acetic acid may be varied rather widely. Theoretically it might appear desirable for most complete heat coagulation to employ a buffer solution of pH 4 to 5. Probably with this end in view dilute mixtures of acetic acid and sodium acetate have been used by Autenrieth and Funk (10) and Mukai (11), while Hohlweg (12) and Richter-Quittner (13) employed mixtures of acetic acid and acid potassium phosphate for the same purpose. Since it was found by trial that 0.01 N acetic acid gave excellent coagulation under the conditions adopted, the use of a buffer was not attempted.

It did appear likely, however, that neutral salts would facilitate extraction of the amino acids (when in high concentration by exosmosis) and assist coagulation of the proteins. Neutral salts, usually sodium chloride or sodium sulfate, as aids in heat coagulation and extraction were used by Liborius (4), Freund and Laubender (14), Fischer (15), Richter-Quittner (13), and many others. We investigated the effect of sodium chloride and calcium chloride additions but with results which showed their use to be unnecessary if not even inadvisable in this method. The amino acid values obtained on the samples treated with neutral salts were no greater than the control. Trouble was also experienced in the cases of high salt concentration through crystallization of the salt during evaporation of the filtrates.

The time of heating was fixed rather arbitrarily at 7 minutes. By gross observation of the tubes, coagulation appeared to be complete within 2 or 3 minutes. Long heating is undesirable because of the expenditure of time and the danger, small though it may be, of protein hydrolysis.

Treatment with Trichloroacetic Acid.

A portion of the tissue protein usually fails to be coagulated and must be removed by other means. For this purpose a great variety of precipitants has been applied to protein-containing fluids, frequently without any preliminary coagulation of the albumins and globulins. Uranium acetate was introduced by Kowalewsky (16) but has not been very extensively used. Sublimate (17) in acid solution enjoyed great popularity for 15 or 20 years. Other metallic salts, such as copper sulfate (18), lead acetate (19), and mercuric chloride (20), have been used considerably. Of the various alkaloidal reagents picric acid, meta-

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phosphoric acid, phosphomolybdic acid, tannic acid—the latter being of particular service as a peptone precipitant—and phosphotungstic acid for more extensive precipitation have been widely used. Tungstic acid (21) has gained universal usage within recent years.

Finally there are three volatile precipitants which might appear to be most satisfactory, since in view of concentrating the filtrate to one-twentieth or less of its original volume it is desirable that an excess of the precipitant be capable of ready removal. These are ethyl alcohol, methyl alcohol, and trichloroacetic acid. The first has been used more extensively than any other protein precipitant. It has been of special value as a solvent for urea in the analysis of tissues for this substance. The use of methyl alcohol was instituted by Folin and Denis (1). Owing, however, to the precipitation of amino acids by these alcohols (1, 3–6), their use in the determination of amino acids in tissues does not appear advisable.

Trichloroacetic acid, on the other hand, is a specific protein precipitant. Because of its sensitivity it was first recommended for the detection of protein in urine (22) and rapidly gained favor as a reagent for blood and tissue analysis. It is a quantitative precipitant of the albumins and globulins and apparently does not precipitate proteoses, peptones, or polypeptides (23).

Relatively low concentrations of trichloroacetic acid (2 to 4 per cent) are effective. We investigated the effect of higher concentrations but with the development of serious difficulties in the later stages of the procedure, which rendered their use undesirable. The 35 cc. portions of filtrate containing 10 to 15 per cent of trichloroacetic acid spattered badly during concentration. Much of the excess trichloroacetic acid failed to be destroyed, during evaporation, and gave a final concentrate of rather high specific gravity. On being transferred to the Van Slyke pipette it settled in the narrow portion above the drainage tap where it failed to mix with the nitrous acid. The results obtained were low, not because of an increased precipitation by higher concentrations of trichloroacetic acid but because of failure of the concentrate to react in the Van Slyke apparatus. This explanation probably applies to a difficulty of a similar nature encountered by Bock (6). The time of precipitation (30 minutes) is that used by Greenwald (5).

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Treatment with Infusorial Earth.

Infusorial earth is used to facilitate filtration and to remove some nitrogenous substances which react with nitrous acid but are not simple amino acids. They are probably proteoses and peptones principally. In the absence of infusorial earth higher amino nitrogen values are obtained. That this is not due to the adsorption of amino acids by infusorial earth is to be inferred from the quantitative recovery of added amino acids and the observations of Michaelis, Pincussohn, and Rona (24), with mastic and kaolin. It is known, nevertheless, that these and other acid silicates are excellent adsorbents of proteins, proteoses, and peptones (25). Alumina cream and colloidal iron are other adsorbents commonly employed to the same end.

Treatment of the Filtrate.

The muscle filtrates have always been observed to be clear and colorless. The liver filtrates are always slightly yellow. If glycogen is present, they are faintly opalescent.

The original purpose of boiling for 2 minutes or so in alkaline solution was to remove ammonia arising from the hydrolysis of urea. Bock (6) added urease in the form of soy bean meal to the blood samples to bring about this hydrolysis. It is doubtful whether the precaution is desirable. Soy bean meal and most of the partly purified urease preparations contain appreciable quantities of amino nitrogen. We are inclined to agree with Blau (7) that the use of soy bean meal in this connection is not advisable. Although we have omitted, therefore, the destruction of urea, we have found it necessary to retain Bock's practice of boiling for a brief period in an alkaline solution. Ammonia, 13 to 17 mg. per 100 gm. of tissue, is given off. It will also be observed that the amino nitrogen values are unduly high if this precaution be omitted.

EXPERIMENTAL.

In much of this work the thigh muscles of rabbits were employed. Elsewhere livers and thigh muscles of albino rats were used. After 24 hours fasting they were killed by stunning and bled by decapitation (rabbits) or a deep incision through the thorax (rats).

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Experiment I. Extraction with Neutral Salts.—To 3 gm. portions of the frozen powdered muscle of a young rabbit were added 5 cc. of a solution of sodium chloride or calcium chloride of the strength indicated. After 5 minutes at room temperature, the suspension was transferred to the dilute acetic acid and boiled for 7 minutes. The estimations were continued according to the method described. The results are presented in Table I.

Experiment II. Prompt Excision of Tissue Is Advisable.—Samples weighing 4 to 5 gm. were excised from the thigh muscles of an adult rabbit at intervals after killing of 2, 10, 18, and 33 minutes, respectively. The corresponding amino nitrogen values expressed in mg. per 100 gm. of tissue were 42.3, 45.1, 48.5, 47.8.

Experiment III. Treatment with Trichloroacetic Acid.—(*a*) Liver and muscle of an adult rabbit were used, and the routine pro-

TABLE I.

Salt solution.	Amino N, mg. per 100 gm. muscle.
Routine procedure	23.5
1 per cent sodium chloride	23.3
10 " " " "	23.4
Saturated " "	20.4
1 per cent calcium "	22.5
Saturated " "	23.4

cedure was altered by adding to the heat-coagulated 3 gm. portions, trichloroacetic acid in final concentrations of 3, 6, 9, and 12 per cent, respectively, and omitting the treatment with infusorial earth. The corresponding amino nitrogen values in mg. per 100 gm. of tissue were: muscle, 45.5, 45.8, 35.8, 19.0; liver, 55.6, 48.9, 43.7, 33.4.

(*b*) From the livers and thigh muscles of four male rats pooled samples of frozen liver and muscle, 15.16 and 15.15 gm. respectively, were weighed out. 150 to 175 cc. of the dilute acetic acid were employed in heat coagulation, trichloroacetic acid was added in a final concentration of 3 per cent, and each was diluted to 250 cc. After 30 minutes standing, infusorial earth being omitted, the samples were filtered. To 40 cc. portions of the respective filtrates trichloroacetic acid was now added in increasing quantities. Each portion was then diluted to 52 cc. and after a further

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30 minutes the newly formed precipitate which was very slight in quantity was removed by centrifuging. In this way the effect of trichloroacetic acid in concentrations of 3, 6, 9, 12, and 15 per cent was determined. The corresponding amino nitrogen values in mg. per 100 gm. of tissue were: muscle, 61.1, 58.2, 50.3, 41.4, 23.9; liver, 42.3, 34.4, 28.9, 22.7, 20.0.

(*c*) 15 gm. samples of rabbit liver and muscle were treated as in Experiment III, b except that infusorial earth was used after the first addition of trichloroacetic acid. When further quantities of trichloroacetic acid (up to 15 per cent) were added to portions of the resultant filtrates, no precipitates formed. The fluids remained perfectly clear. The material which is capable of precipitation by higher concentrations of trichloroacetic acid may also be adsorbed by infusorial earth.

(*d*) 15 gm. samples of rat liver and muscle were treated as in Experiment III, b. No precipitate formed when increasing quantities of trichloroacetic acid were added to portions of the respective filtrates. Nevertheless those portions now containing trichloroacetic acid in concentrations of 3, 6, 9, 12, and 15 per cent respectively were evaporated in the usual manner. The corresponding amino nitrogen values expressed in mg. per 100 gm. of tissue were: muscle, 50.0, 52.5, 49.3, 32.3, 36.4;[1] liver, 44.7, 44.2, 34.5, 32.2, 20.9.

(*e*) A not unlikely explanation of the amino nitrogen decrease observed in Experiment III, d would be through anhydride formation, for as the concentrate became progressively more dense in the higher concentrations of trichloroacetic acid, the boiling point would be higher, and anhydride formation would proceed more rapidly. This possibility was now examined.

Three solutions of the following compositions were prepared: (A) 1 cc. of an amino acid solution (1.22 mg. of amino nitrogen) obtained by the total hydrolysis of egg albumin; 10 cc. of 50 per cent trichloroacetic acid; 22 cc. of water. (B) 1 cc. of the amino acid solution; 2 cc. of 50 per cent trichloroacetic acid; 30 cc. of water. (C) 5 cc. of the amino acid solution; 10 cc. of 50 per cent

[1] The Van Slyke pipette was shaken occasionally while the concentrated sample was run in from the side burette. This produced a partial mixing of the materials and decreased the degree of settling of the concentrate in the bottom of the pipette.

trichloroacetic acid; 18 cc. of water. These solutions were concentrated in the usual way, Solutions A and B being reduced to about 2 cc. and transferred *in toto* to the Van Slyke apparatus, Solution C being similarly reduced but finally made up to exactly 10 cc. with water. Of the latter, 2 cc. samples were used for analysis in the Van Slyke apparatus. The analytical results were as follows: (A) 15 per cent trichloroacetic acid, 0.57 mg. of amino nitrogen, 47 per cent recovery; (B) 3 per cent trichloroacetic acid, 1.22 mg. of amino nitrogen, 100 per cent recovery; (C) 15 per cent trichloroacetic acid, 1.17 mg. of amino nitrogen, 96 per cent recovery. Solution A settled in the bottom of the Van Slyke pipette; Solutions B and C did not. Experiments III, a and III, e demonstrate quite clearly that trichloroacetic acid may not be used in concentrations greater than 6 per cent (Experiment III, d) in this analytical method.

Experiment IV. Treatment with Infusorial Earth.—15 gm. samples of frozen rat liver and muscle were suspended in 150 to 175 cc. of the boiling dilute acetic acid. After heat coagulation the suspensions were treated with trichloroacetic acid in a final concentration of 3 per cent and diluted with water to 250 cc. To 50 cc. portions of the respective filtrates, the following quantities of infusorial earth were added: 0.0, 0.5, 1.5, 3.0 gm., respectively. After 10 to 15 seconds of vigorous shaking, the mixtures were filtered. The amino nitrogen values corresponding respectively to the above quantities of infusorial earth were as follows: muscle, 68.1, sample lost, 51.8, 48.4; liver, 45.9, 38.2, 33.8, 33.9.

We concluded from these results that of the material removed by the infusorial earth, some contains amino nitrogen. 2 gm. of the adsorbent appear to be sufficient.

Experiment V. Concentration of the Filtrate.—(*a*) Of the filtrate obtained from rabbit muscle, equal portions were taken and concentrated to about 10 cc. They were then made alkaline in the usual manner and boiled for varying times; *viz.*, 0.0, 0.1, 0.5, 1.0, 2.0, 4.0 minutes. At the ends of the specified intervals the fluids were acidified with acetic acid and the evaporation continued. The corresponding amino nitrogen values were 69.1, 60.0, 56.2, 54.9, 57.0, 57.1.

This indicates the presence in the filtrates of a readily volatile base (probably ammonia). The quantity, judging from the amino nitrogen values, is considerable.

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(*b*) 2, 4, 6, and 8 gm. samples of rabbit muscle were each treated with 30 to 35 cc. of hot dilute acetic acid. The suspensions were boiled for 7 minutes, cooled, and treated with trichloroacetic acid. The volumes were made up to 50 cc. and filtered following the 30 minute interval for precipitation and the addition of infusorial earth. The filtrates were concentrated directly to 2 cc., except that of the 6 gm. sample which received the 2 minute boiling in alkaline solution. The amino nitrogen values expressed in mg. per 100 gm. of tissue were: 2 gm. sample, 47.1; 4 gm. sample, 47.0; 6 gm. sample, 25.2; 8 gm. sample, 44.7. This confirms the observation reported in Experiment V, a above.

(*c*) 40 cc. portions of the filtrates obtained by the routine procedure from 15 gm. samples of rat liver and muscle were concentrated to about 10 cc. Two liver and two muscle samples were made alkaline for 2 minutes, boiled, reacidified, and concentrated to about 2 cc. Two liver and two muscle samples were concentrated directly to 2 cc. without heating in alkaline solution.

	Amino N per 100 gm. tissue.
	mg.
Liver filtrate.	
Boiled in alkaline solution	37.8
" " " "	37.4
Not boiled in alkaline solution	56.7
" " " " "	56.5
Muscle filtrate.	
Boiled in alkaline solution	48.1
" " " "	48.8
Not boiled in alkaline solution	59.3
" " " " "	59.3

(*d*) In the case of sixteen rats the ammonia given off during the 2 minutes boiling in alkaline solution was collected and estimated by Nesslerization.

No. of samples.	Ammonia N, mg. per cent.		
	Average.	Minimum.	Maximum.
16 muscle.	12.8	11.9	14.2
15 liver.	17.2	15.3	18.7

(*e*) Owing to the fact that the ammonia values were of the same magnitude as accepted values for the urea nitrogen content of

tissues, we wondered whether the ammonia arose from total hydrolysis of tissue urea.

To 3 gm. samples of frozen rabbit muscle increasing quantities of urea were added. The routine procedure already described was then followed. The ammonia given off during concentration of the filtrates was collected and estimated.

	Ammonia N, mg. per 13 gm. tissue.	
	Found.	Calculated.*
No urea added	0.435	
1 cc. urea solution added	0.448	0.660
2 " " " "	0.492	0.885

* Assuming all of the added urea to be hydrolyzed.

It appears therefore that the ammonia is not formed by the destruction of urea in the course of the analysis.

(*f*) The ammonia given off may not represent preformed tissue ammonia. (1) Ammonia reacts slowly with nitrous acid. The 2 minute boiling in alkaline solution removes 13 to 17 mg. per cent of ammonia N (Experiment V, d) but also causes a decrease in the amino nitrogen of 11 to 19 mg. per cent (Experiment V, c). (2) Blood, which contains a negligible quantity of ammonia, suffers the same decrease in the amino nitrogen value as a result of 2 minutes boiling of the filtrate in alkaline solution.

Dog blood was treated according to the routine procedure described for muscle and liver. Freezing with liquid air was omitted. Of two portions of the filtrate, one was concentrated to 2 cc. directly, the other was submitted to the routine treatment with sodium hydroxide. The latter gave an amino nitrogen value of 8.0 mg. per cent (the accepted order of magnitude for blood amino nitrogen); the former gave a value of 16.4.

Experiment VI. Recovery of Added Amino Acids.—(*a*) To four 2 gm. portions of frozen rabbit muscle were added 0.0, 1.0, 2.0, and 3.0 cc. respectively, of an aqueous solution of *dl*-alanine (0.254 gm. per 100 cc.). The samples were then transferred to boiling tubes with the hot dilute acetic acid. The rest of the procedure was according to the method described. The following amino nitrogen values expressed in mg. per 2 gm. of muscle were obtained: 0.819,

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1.125, 1.532, 1.848. The corresponding calculated values were: 0.819, 1.219, 1.619, 2.019. Per cent recovery, 93, 94, 92.

(*b*) The above experiment was repeated with a solution of monoamino monocarboxylic acids obtained by butyl alcohol extraction of totally hydrolyzed casein (26) (0.405 gm. of monoamino acids (0.038 gm. of amino N) per 100 cc.). Amino nitrogen values obtained: 0.673, 1.022, 1.358, 1.743; calculated, 0.673, 1.059, 1.442, 1.816; per cent recovery, 96, 94, 96.

Experiment VII. Amino Nitrogen Content of Rat Liver and Muscle.—(*a*) Five female rats (150 to 180 gm. in weight) were fasted for 24 hours. They were then killed by stunning and bleeding through a thoracic incision. The liver and the thigh muscles of one side were rapidly excised and analyzed according to the routine procedure. The following amino nitrogen values expressed in mg. per 100 gm. of tissue were obtained: liver, sample lost, 40.3, 43.2, 36.6, 43.8; muscle, 51.1, 50.5, 57.6, 50.3, 49.3.

(*b*) Five female rats (150 to 170 gm. in weight) were similarly treated. Infusorial earth was omitted. The higher values which follow were obtained: liver, 45.6, sample lost, 47.2, 51.2, 51.9; muscle, 59.3, 59.7, 57.5, 61.1, 59.3.

Part of this work was done in the Biochemical Laboratory of the University of Toronto.

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THE METABOLISM OF AMINO ACIDS.

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(From the Department of Chemistry, Stanford University, California.)

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It was shown by Seth and Luck (1) that the intestinal absorption of glycine and alanine by rabbits was followed by pronounced aminoacidemia. Other amino acids when administered in similar amounts provoked smaller increases in the amino nitrogen content of the blood. Glutamic acid and aspartic acid were absorbed with but a slight aminoacidemia.

The question then arose as to whether these striking differences in behavior were due to equally profound differences in the rates of absorption from the alimentary canal, in the rates of diffusion into liver, muscle, and other tissues, and in the rates of metabolism of the amino acids therein. It may be recalled that Bang (2) demonstrated marked inequalities in the absorption of various amino acids by isolated fragments of liver suspended in solutions of these substances. A not unlikely explanation of the results of Seth and Luck would seem to be that the glycine-alanine aminoacidemia was due to slow diffusion of the substances into liver and muscle, or a low rate of oxidation of the metabolites, or both. In contrast, the failure of glutamic and aspartic acids to provoke an appreciable increase in the amino acid content of the blood might be attributed to their rapid absorption therefrom.

According to such a theory it might be expected that the ingestion of glycine and alanine would cause but a small increase in the amino acid content of liver and muscle, or a slow conversion of the amino nitrogen to urea. On the other hand glutamic acid might be expected to increase more markedly the amino acid content of liver and muscle or to lead to more rapid urea formation than is observed with glycine and alanine. Finally one may advance the view that the differences in the degree of aminoacidemia may be due in part to specific differences in the excretion of amino acids by the kidney.

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In this paper a report is given of changes in the amino acid content of blood, liver, and muscle following the oral administration of amino acids. The object of the work was to determine the relationship if any which existed between the degree of aminoacidemia obtained and the concentration increase of the amino acids in liver and muscle. The findings proved to be of a somewhat unexpected nature.

EXPERIMENTAL.

Male albino rats of 160 to 220 gm. in weight (except in Experiments 55 and 58) were employed throughout. The actual weights of the animals in gm. on the day of experiment were as follows: Fxperiment 19, 160, 164, 180, 164; Experiment 21, 188, 206, 188, 196; Experiment 23, 200, 180, 188, 180; Experiment 25, 198, 220, 188, 190; Experiment 27, 210 to 220; Experiment 29, 194, 190, 198, 199; Experiment 31, 210 to 218; Experiment 33, 190 to 210; Experiment 35, 200 to 220; Experiment 39, 200 to 220; Experiment 41, 190 to 210; Experiment 44, 160 to 170; Experiment 48, 200 to 210; Experiment 50, 180 to 200; Experiment 52, 155 to 160; Experiment 55, 120 to 151; Experiment 58, 128 to 147.

The animals were maintained on a standard diet[1] and fasted for 24 hours preceding an experiment. The animals were used in groups of four or five. The experimental material was administered by stomach tube (a No. 8 French catheter was used) after which the animals were killed at intervals of approximately 0, 0.8, 2, 4, and 6 hours from the time of administration. The rats were killed by stunning, and rapid incision of the thorax. The blood which drained away was collected in a crucible containing powdered potassium oxalate.[2] The liver and 4 to 5 gm. of muscle

[1] Cracked wheat 26, oatmeal 26, corn-meal 26, flaxseed meal 10, dried whole milk (Klim) 5, alfalfa (leaves and blossoms) 5, bone meal 15, sodium chloride 0.5,—with greenstuffs two or three times per week.

[2] It was found necessary to stun the animal by a sharp blow in the mid-cervical region. If the blow were received on the head, profuse bleeding took place through the nose. Such blood was rejected in view of the possibility that it might have come in contact with the mouth which would probably contain drippings from the stomach tube. If the blow were received in the thoracic region, intravascular blood clotting was observed to proceed with such rapidity that only a drop or two of semiclotted blood would drain from the incision.

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from the hind limbs were promptly excised and frozen by immersion in liquid air. The amino nitrogen contained in these samples was determined by the method described in the preceding paper (3). The amino nitrogen content of the blood was determined by the method of Folin (4).

The dicarboxylic acid fraction of hydrolyzed caseinogen, the monoamino monocarboxylic acid fraction of hydrolyzed caseinogen, totally hydrolyzed egg albumin, *dl*-aspartic acid, *d*-glutamic acid, glycine, and *dl*-alanine, were used. The last four were obtained from the Eastman Kodak Company. From acid-hydrolyzed caseinogen the monoamino monocarboxylic acids were obtained by the butyl alcohol extraction method of Dakin (5), and the dicarboxylic acids by precipitation of the calcium salts (6) from the non-extractable portion. The residue represented the hexone base fraction. The sulfuric acid-barium hydroxide method was employed in hydrolysis of the egg albumin.

Each rat received 3 cc. of an aqueous, neutralized, solution of the experimental substance equivalent in concentration to 0.2, 0.3, or 0.4 gm. of amino nitrogen per kilo.

It will be seen that by this procedure any questionable secondary effects resulting from prolonged anesthesia and the use of surgical methods are avoided. The animal is in a normal state throughout the experiment. The metabolism of the amino acids proceeds normally in every tissue. Within 2 minutes of the killing of the animal the samples are frozen and postmortem autolytic changes are prevented.

It is also apparent that the method of group experimentation permits one to make a number of successive analyses at suitably spaced intervals of time. This cannot be done by the single animal method without employing anesthetics and resorting to troublesome if not questionable operative means. It is evident moreover if smooth continuous curves be obtained by the series method when time is plotted against the tissue concentration of the metabolite, that each animal serves as a confirmatory check against its neighbors in the series. It is almost unnecessary to point out that the use of small animals is no mean consideration when costly amino acids are to be administered.

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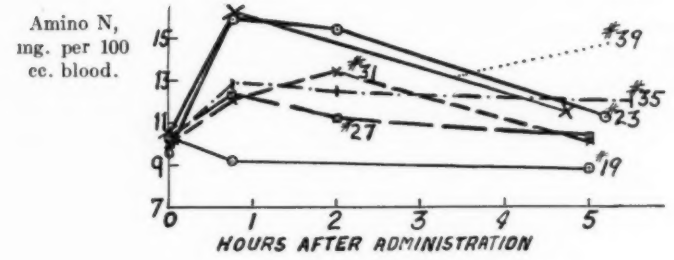

CHART 1A.

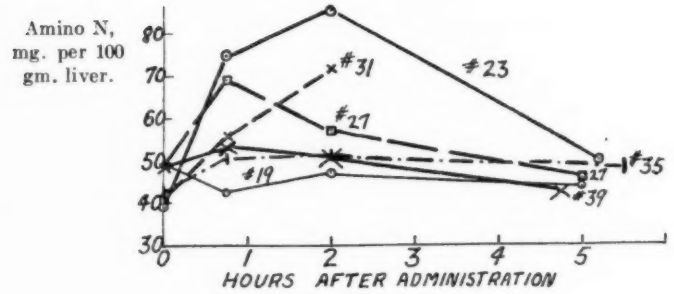

CHART 1B.

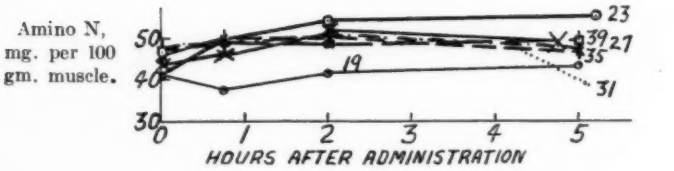

CHART 1C.

CHART 1. Experiment 19, water; Experiment 23, glycine; Experiment 27, dicarboxylic acids (⊡—— ——⊡); Experiment 31, hexone bases (×— — —×); Experiment 35, monoamino acids (—·—·—·—); Experiment 39, alanine (×———×).

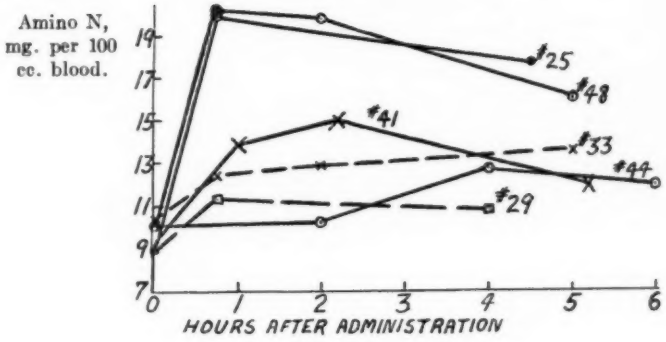

CHART 2A.

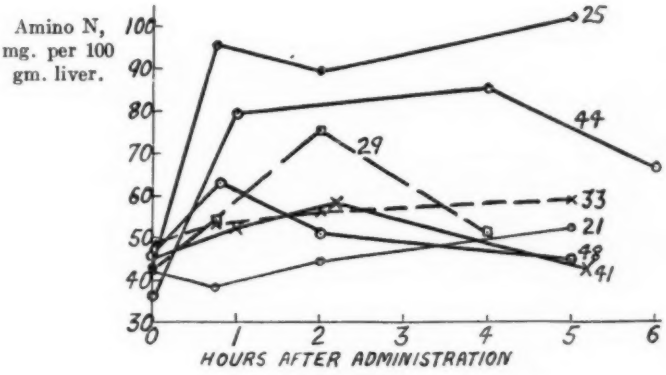

CHART 2B.

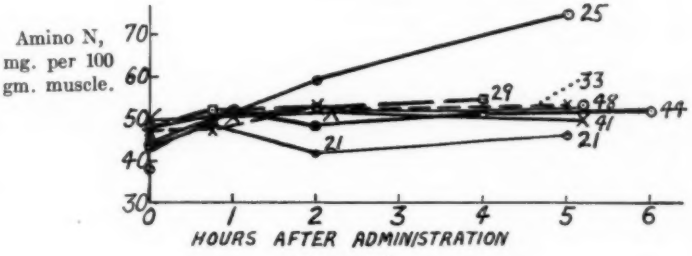

CHART 2C.

CHART 2. Experiment 21, water; Experiment 25, glycine; Experiment 29, dicarboxylic acids; Experiment 33, hexone bases; Experiment 41, alanine; Experiment 44, totally hydrolyzed albumin; Experiment 48, alanine.

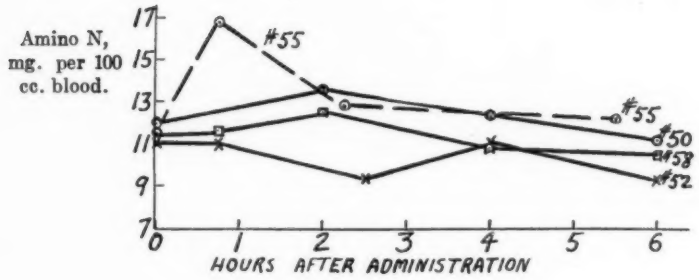

CHART 3A.

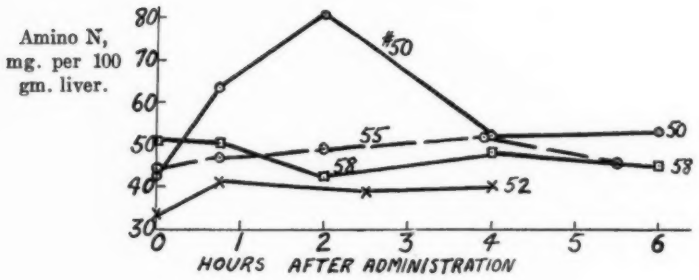

CHART 3B.

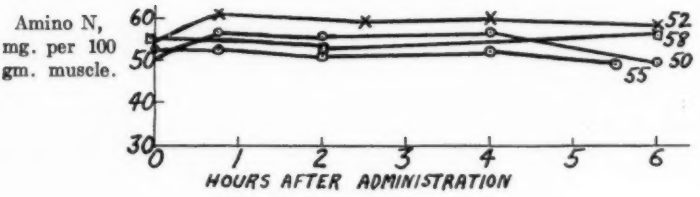

CHART 3C.

CHART 3. Experiment 50, totally hydrolyzed albumin; Experiment 52, aspartic acid; Experiment 55, monoamino acids; Experiment 58, glutamic acid.

Results.

Amino Acid Content of Blood, Liver, and Muscle.—The accompanying curves are self-explanatory. Charts 1 A, 1 B, 1 C repre-

TABLE I.

Ammonia Content of Liver and Muscle.

Experimental substance.	Tissue.	Ammonia N, mg. per 100 gm. tissue. Time after administration.				
		0 hr.	½-1 hr.	2-2½ hrs.	4-4½ hrs.	5-6 hrs.
Water.	Muscle.	15.0	12.5	12.1		11.9
	Liver.	18.5	18.6	15.3		15.9
Water.	Muscle.	13.7	14.2	14.1		12.6
	Liver.	17.4	16.1	17.7		17.2
Glycine, 0.19 gm. N per kilo.	Muscle.	13.2	13.2			13.4
	Liver.	19.5	16.9	16.2		17.3
Glycine, 0.38 gm. N per kilo.	Muscle.	10.8	11.5	12.1		
	Liver.		14.4	14.6		
Dicarboxylic acid fraction, 0.20 gm. N per kilo.	Muscle.	12.5	14.9	13.6		12.9
	Liver.	18.7	21.6	22.5		19.7
Dicarboxylic acid fraction, 0.40 gm. N per kilo.	Muscle.	12.9	11.6	12.6	13.6	
	Liver.	16.3		19.6		
Hexone base fraction, 0.20 gm. N per kilo.	Muscle.	11.9	13.0	11.1		14.0
	Liver.	14.3	17.3	17.3		15.7
Hexone base fraction, 0.80 gm. N per kilo.	Muscle.	12.6	14.4	14.9		14.2
	Liver.	18.4	20.6	20.6		22.0
Monoamino acid fraction, 0.20 gm. N per kilo.	Muscle.	12.4	13.0	13.0		12.6
	Liver.	16.9	16.5	17.9		16.0
Alanine, 0.20 gm. N per kilo.	Muscle.	12.9	13.2	14.5		13.3
	Liver.	17.1	18.9	17.8		14.5
Alanine, 0.40 gm. N per kilo.	Muscle.		12.8	13.5		15.0
	Liver.		17.0	16.2		16.9

sent the results of experiments in which the substances were administered in quantities of 0.20 gm. of amino nitrogen per kilo

of rats. In Charts 2 A, 2 B, 2 C the quantities were 0.40 gm. of amino nitrogen, and in Charts 3 A, 3 B, 3 C, 0.30 gm. of amino nitrogen per kilo. There are, however, the following exceptions:

Experiment No.	*gm. per kg.*
23	0.19, amino N
25	0.38, " "
33	0.80, total "
44	0.47, amino "

Ammonia Content of Liver and Muscle.—It proved convenient in some of the experiments to determine the amounts of ammonia evolved during the period of boiling in alkaline solution required by this analytical method. The ammonia was collected in dilute sulfuric acid and estimated by Nesslerizing. The results are presented in Table I.

The ammonia content of liver and muscle showed no appreciable change or but slight increases following the absorption of amino acids. The absolute ammonia values were invariably greater in liver than in muscle, being 14.3 to 22.5 mg. per cent in liver and 10.8 to 15.0 mg. per cent in muscle. These values agree approximately with those reported by others (7). We are not satisfied, however, that the methods employed in this work or in that of any others known to the writer, give ammonia values of much significance. It is probable (*cf.* Gad-Andresen, 1919; Warburg *et al.*, 1924; Meyerhof, 1925; Grafe, 1925) that the same scrupulous care needs to be exercised in such estimations as is known to be indispensable in determining the ammonia content of blood.

DISCUSSION.

The most striking result of these experiments is to be seen in the absence of any appreciable increase in the amino nitrogen content of muscle following the oral administration of amino acids.

To this generalization, glycine alone is exceptional. Following the oral administration of this substance the amino nitrogen content of muscle increases slowly and uniformly, this increase proceeding at an unchanged rate after the maximum increases in the blood and liver have been observed (Charts 2 A, 2 B, 2 C). Whatever additional significance these relative rates of change may have, certain it is that the accumulation of glycine in muscle

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proceeds much more slowly than it does in the liver. This is doubtless accountable in part by the method of administration, —all of the absorbed amino acids having to pass through the liver before entering the systemic circulation. It is not even improbable that the pronounced difference in the behavior of the whole group of amino acids in liver and muscle is to be explained similarly. We are examining this point experimentally by administering these substances in other ways.

It is perhaps well to point out that the amino nitrogen increase in muscle is not determined by the level of amino acids in blood. This follows from the results obtained with alanine which caused an equally marked aminoacidemia but no apparent increase in muscle amino nitrogen. Nor is there any relationship between the amino acid increase in liver and the behavior in muscle. Totally hydrolyzed egg albumin increased the amino nitrogen content of the liver almost as greatly as did glycine, but resulted in no appreciable change in the amino acid content of muscle (Charts 2 B, 2 C). The behavior of glycine in muscle is apparently specific for that amino acid. It cannot be considered a representative member of the products of protein hydrolysis and the deduction of generalizations in protein metabolism from results obtained primarily if not solely with glycine is to be cautioned against.

The remarkable differences in behavior of glycine and alanine were quite unexpected. In view of their neighborly relationship in a homologous series and the similar increases in the amino nitrogen content of blood following their oral administration, it was considered likely that they would induce the same measure of change in liver and muscle. It is to be observed, however, that while glycine induced a marked increase in the amino nitrogen content of the liver, alanine caused but a slight and transient increase. The contrast in their behavior in muscle has already been pointed out. It is not likely, moreover, that results materially different would have attended the use of *d*-alanine instead of the racemic mixture. For though it might be supposed that *d*-alanine would be metabolized more or less rapidly than *l*-alanine, it is very improbable that any pronounced increase in liver and muscle amino nitrogen caused by one isomer would be completely nullified by the other. It is to be observed in this connection that the naturally occurring isomer of glutamic acid behaved qualitatively in muscle like the racemic alanine (Charts 2 C, 3 C).

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It will have been noticed that though in muscle none of the amino acids except glycine increased the amino nitrogen content of that tissue, most of them elevated in some measure the amino nitrogen content of the liver. This in itself throws little light on the relative dominance of the rôles of liver and muscle in protein metabolism. As has already been mentioned, the mode of administration of the experimental material almost certainly determines in part the muscle-liver picture. It might appear permissible, moreover, to regard muscle as being so efficient in the metabolism of absorbed amino acids that it succeeds in maintaining its amino nitrogen content at the normal level. This sort of explanation is discredited by the vast amount of evidence, much of it of great weight, concerning the locus of amino acid catabolism. Most of this regards but poorly the urea-forming power of muscle. As for the anabolic change, the rapid synthesis of protein from the absorbed material, it is difficult to see how this could proceed from a single amino acid. Finally, a quite improbable explanation would be to regard muscle as being impermeable to all amino acids but glycine. One would then be driven to exercise unusual ingenuity to account for the formation of the muscle proteins.

It is somewhat premature to advance an explanation for the profound inequalities in the rates of increase of the various amino acids in blood and liver. Additional information must first be had concerning the excretion of these substances by the kidney, their rates of oxidation, and the differences if any in their rates of absorption from the alimentary canal. With respect to the last mentioned point we have reason to believe that glycine, alanine, glutamic acid, and aspartic acid are absorbed at much the same rate from an isolated intestinal loop of the dog (1). It will also be noticed that all of the amino acid preparations employed in this work are readily soluble in water,—a consideration which is pertinent to the question of their absorption.

A discussion of these experiments cannot be complete without reference to the closely related work of others.

It appears as a first consideration that the amino acid content of animal tissues fluctuates normally within rather narrow limits. Fasting appears to be without effect, as demonstrated by Van Slyke and Meyer (8) on dogs and by Mitchell, Nevens, and Kendall (9) on the entire carcasses of rats after fasting for 19 to

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26 hours. We too have never observed any change in the amino acid content of the whole animal or in the muscles and livers of rats as a result of fasting 1 or 2 days. Buglia and Costantino (10) in a few experiments on dogs observed slightly higher values for the non-urea, non-protein nitrogen of muscle after fasting periods of 12 to 25 days. It does not necessarily follow, however, that the amino acids were similarly increased in quantity. The analytical method employed by these investigators (desiccation of the samples at 70–80° and formol titration of the extract) is also open to criticism.

Neither do high nor low protein diets appear to alter very markedly the concentration of amino acids in tissues. Mitchell, Nevens, and Kendall report experiments of 11 to 48 days duration in which rats were maintained on nitrogen-free diets without

TABLE II.

Amino Nitrogen Content of a Foreign Group of Rats.

Rat No.	Amino N, mg. per 100 gm. tissue.	
	Muscle.	Liver.
1	78.9	70.9
2	76.0	66.5
3	72.6	68.7

change in the amino acid content of the animals. Kiech (unpublished data) in this laboratory has observed that high and low protein diets administered to rats for 2 day periods are productive of small but certain differences. Thus twenty-six rats which had been on a starch-butter diet for 2 days contained an average of 47.4 mg. of amino nitrogen per 100 gm. of tissue. All values fell between 40.2 and 51.8. Similarly nine rats on a high protein diet (87 per cent caseinogen) for 2 days gave corresponding values of 50.6 (47.1 to 53.5).

There is, however, an indication as shown in Table II that some factors presumably of dietary origin may influence profoundly the amino acid content of liver and muscle. The group of rats reported in Table II was of the same age, size, and sex as the animals used in all the other experiments. They had been fasted for 24 hours before analysis. They came from another colony and

were related as second or third cousins to one section of our own stock. They had, however, been maintained since weaning on a very different and much more varied basal diet. This group, the only foreign one examined, has been the only one to give abnormal basal values. Mitchell (7) has also reported on adult rats which gave, so it seems to us, unusually high amino acid values.

The amino acid content of muscle is increased following hepatectomy (11).

Apart from changes induced by the ingestion of proteins or their products of hydrolysis, the amino acid content of blood is maintained at quite a constant level. In acute yellow atrophy of the liver (12), myelogenous leucemia (13–15), possibly in pernicious anemia (16), in polycythemia (17), and in hydrazine poisoning (18), the amino acid content of the blood is greater than normal. It is also probable that in disturbances of carbohydrate metabolism of pancreatic origin (13, 19), the amino acid content of the blood is altered.

Finally reference may be made to other experiments on the effect of protein or amino acid administration on the amino acid content of muscle and liver. The feeding of large quantities of meat to dogs was found by Wishart (20) to be without influence on the non-urea, non-protein nitrogen of the muscle. This agrees with our own observations on the fate of ingested amino acids. In the well known experiments of Van Slyke and Meyer (21), alanine and hydrolyzed caseinogen were injected intravenously in dogs. Cathcart (22) administered glycine to dogs by the same means. Although the results of these experiments are in agreement with our own in so far as they indicate changes of greater magnitude and rapidity in the amino acid content of liver than in muscle, they differ with respect to the absolute increases observed. This is, however, to be expected in view of the different mode of administration of the experimental materials, and the important differences in subsequent treatment of the animal and analysis of the tissue samples.

The interesting experiments of Lombroso, Artom, Paterni, and Luchetti (23) on the entrance of amino acids into the perfused liver, muscle, and kidney are hard to interpret because of the conflicting results obtained with defibrinated blood and Ringer's solution respectively.

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Experiments similar in method are now in progress to study the entrance of the amino acids into liver and muscle after subcutaneous injection, and to determine the differences, if any, in their rates of oxidation, *in vivo*.

Part of this work was done in the Biochemical Laboratory of the University of Toronto.

SUMMARY.

1. When amino acids are administered to rats, *per os*, and in equimolecular amounts, increases of varying magnitude are observed in the amino acid content of liver, but no appreciable change, except with glycine, is observed in the amino acid content of muscle.

2. Although glycine and alanine increased in the same measure the amino acid content of the systemic blood, the former provoked a great increase in the amino nitrogen content of liver, while the latter caused no significant change.

3. In most cases no appreciable changes were observed in the ammonia content of liver and muscle.

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XUM

GROWTH ON A SYNTHETIC RATION CONTAINING SMALL AMOUNTS OF SODIUM.*

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(Received for publication, November 28, 1927.)

The effect of individual inorganic elements in the diet has received increasing attention during the last few years. This is true of sodium and potassium, to which very little attention has been given since the work of Bunge (1873). Osborne and Mendel (1918), using a purified diet, concluded that rats could be successfully raised on a diet containing less than 0.04 per cent of sodium or potassium although when both were at this low level growth ceased. Their diet was very high in fat, containing 25 per cent. Their paper does not indicate that their animals were excluded from the feces. Also, the particular element being studied was not the only variable in the ration since when one element was eliminated from the diet, it was replaced by an equivalent amount of others in order to adjust the acid-base balance. Lamb and Evvard (1921) make this statement: "If the other elements in a natural ration are satisfactory, it is not necessary to balance the acid and basic mineral elements for growing swine."

Miller (1923) seems to have obtained satisfactory growth on a synthetic ration, including cod liver oil, containing 0.07 per cent of sodium. His animals were kept on screens. A ration with a K to Na ratio of 14:1 is said to have had no deleterious effect on the growth of young rats. Miller (1926) used a ration which included 80 per cent of corn and contained 0.03 per cent of sodium. This did not promote normal growth. With sufficient sodium carbonate or sulfate to make 0.42 per cent sodium in the diet

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satisfactory growth was obtained. Mitchell and Carman (1926) fed a ration containing 87 per cent corn, including 0.047 per cent sodium, which gave some growth. However, with sodium chloride added to the extent of 1 per cent, much better growth was secured. Their rats were kept in wire cages without bedding. They suggest that the addition of more sodium might have given Osborne and Mendel much better growth.

Palmer and Kennedy (1927), using a ration containing highly purified casein, Salt Mixture 185,[1] agar, butter fat, Crisco, and dextrin, and keeping the rats on screens, failed to get growth. It will be noted that the ration contained 15 per cent fat. Sodium was present to the extent of about 0.126 per cent. Richards, Godden, and Husband (1924) fed a ration of corn, oat, barley, and blood meals to growing pigs. The addition of sodium chloride or citrate led to increased assimilation and retention of nitrogen, calcium, and phosphorus. The ratios of potassium to sodium were 1.62:1 and 1.53:1 during the periods of increased retention. Potassium excretion was affected very little. They feel that their data do not support Bunge's theory and that Miller's data (1923, 1926) support their conclusions rather than Bunge's. These authors (1927) review other work on sodium and potassium.

Following the work carried out by Olson and St. John (1925) in 1920 to 1922 on a wheat ration, a study was made of the amount of sodium required in a synthetic ration. The experimental work on these rations was completed in March, 1925. The diet was modified from the one used by Osborne and Mendel (1918) and contained casein 18, yeast 3, agar 2, lard 5, cod liver oil 1,[2] starch 67, and salt mixture 4. Sodium carbonate was eliminated from the salt mixture. Calcium, magnesium, and potassium carbonates and citric acid were added in powder form. The rest of the salts and the acids were added in solution. To this basic ration sodium bicarbonate was added in varying amounts to furnish rations containing the desired per cent of sodium. All rations were thus constant in composition with the exception of the quantity of sodium bicarbonate used. It is hoped that higher percentages

[1] McCollum, E. V., and Simmonds, N., *J. Biol. Chem.*, 1918, xxxiii, 63.

[2] Freshly procured Eli Lilly and Company highest quality pure Norwegian cod liver oil, biologically tested and containing not less than 600 units of vitamin A in each fluid ounce.

of sodium may be used later. The rats were kept on screens without bedding.

Figs. 1 and 2 show the growth curves of rats fed upon rations containing amounts of sodium varying from 0.02 to 0.5 per cent.

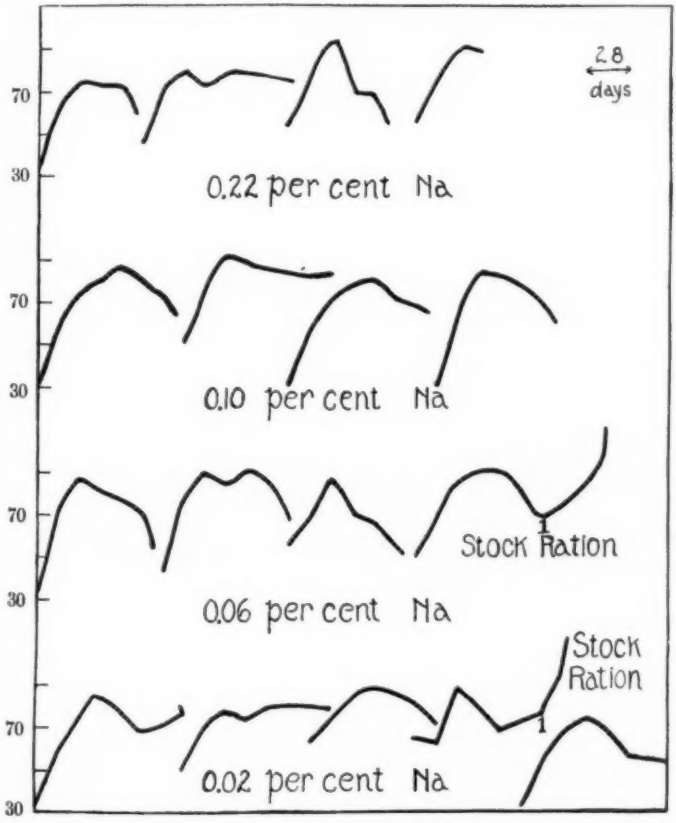

FIG. 1. At the points indicated by the figure 1, the stock ration was added.

In no case was successful growth obtained with rations containing 0.30 per cent or less of sodium. The animals grew rapidly for a few weeks and then declined in weight, many of them dying in

10 to 12 weeks. It will be noted that the peak of the growth curve occurs at 4 to 8 weeks with the rations containing 0.22 per cent or less of sodium, while with the ration containing 0.30 per cent of sodium, the peak of the curves occurs at 10 to 14 weeks. This peak is also higher than with the rations containing smaller amounts of sodium.

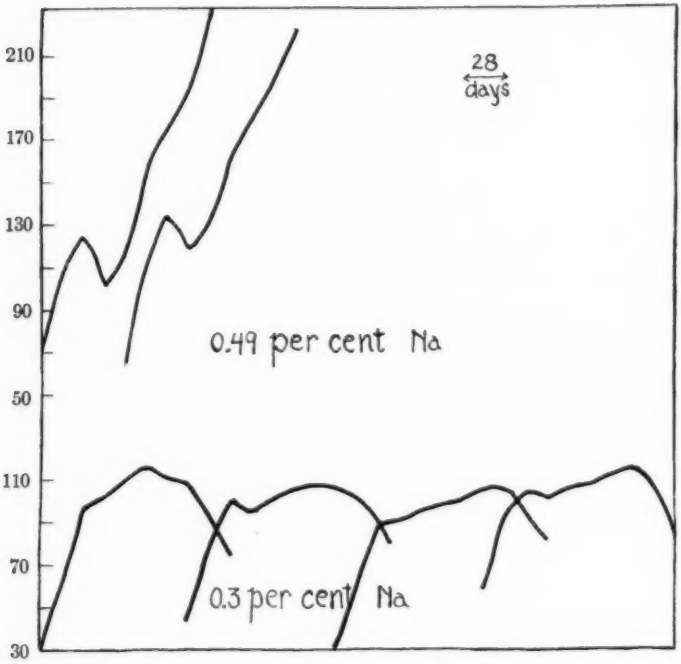

FIG. 2.

The difference in the appearance of the animals was more striking than the differences in the growth curves. Fig. 3 shows animals receiving 0.22 and 0.30 per cent sodium respectively. The rats receiving the larger amount of sodium were in much better condition. The eyes of many of the animals receiving the smaller amounts of sodium were seriously affected,

and blindness resulted in many cases. The eyes of the animals receiving larger amounts of sodium were not affected. There was no reproduction on any of the rations used. This was perhaps influenced by lack of vitamin E. Poor growth was not due to lack of vitamins A, B, or D since several additional rations with double the amounts of yeast and cod liver oil gave the same results as the original rations with the corresponding amounts of sodium. Substitution of the stock ration for the experimental ration invariably caused immediate and rapid growth and improvement of condition, as illustrated in Fig. 1 with two animals.

There are perhaps several reasons why Osborne and Mendel (1918) obtained fairly satisfactory growth on a ration containing

FIG. 3.

less than 0.04 per cent sodium. Their animals were apparently not prevented from practicing coprophagy. The fat content of their diet was far above the amount normally contained in natural foods. This may have affected their results and also those of Palmer and Kennedy (1927). The ratio of potassium to sodium in Osborne and Mendel's control diet was 4.5:1, while in their sodium-free diet it was 23.8:1, and in Palmer and Kennedy's diet the ratio was 3.4:1. Richards, Godden, and Husband (1927) obtained increased retention of nitrogen, calcium, and phosphorus when the above ratio was 1.62:1 and 1.53:1. Olson and St. John (1925) obtain the best results considering both growth and repro-

duction when this ratio was 0.6:1 while with a ratio of 1.4:1 the results were not satisfactory. This ration contained 0.32 per cent potassium. In view of the results of these and other investigators, it will be seen that the best results may in general be expected when the ratio of potassium to sodium is low. However, if we consider the actual amount of these two elements in the various rations used, it appears that the level of sodium in the diet is at least as important as this ratio.

Amounts of sodium below 0.3 per cent proved inadequate for growth. With this amount of sodium growth was improved and the animals appeared in much better condition. Additional sodium was accompanied by improved growth.

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XUM

INSECTS AS TEST ANIMALS IN VITAMIN RESEARCH.

I. VITAMIN REQUIREMENTS OF THE FLOUR BEETLE, TRIBOLIUM CONFUSUM DUVAL.*

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Our increasing understanding of the complexity of food constituents and the nutritional requirements of higher animals might be considerably accelerated if the science had a more complete evolutionary foundation. If, as Bayliss (1) maintains, increasing complexity of organisms in the course of evolution is associated with lessened ability to synthesize the compounds of which they consist, lower animals should be expected to have the simpler and hence more easily understood food needs. Actual knowledge does not permit application of this generalization to the succession of forms in the animal kingdom at this time but the recent work of Wulzen (2) on the nutrition of planarian worms is, no doubt, a forerunner of future ventures into this field.

It is the purpose to present in this paper certain results from a more extended study of the nutritional requirements of the confused flour beetle, *Tribolium confusum* Duval, which seem to prove the value of a phylogenetic view-point in the science of nutrition and the usefulness of this species as an experimental animal in biological analysis. The general plan of the experiments has been a study of the effects on growth of modification of food materials

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by extraction and of rations consisting of purified foodstuffs, with several series and to apply this knowledge in quantitative and qualitative analyses of growth-promoting content.

REVIEW OF LITERATURE.

A few investigators have attempted to determine the nutritional requirements of certain insects, desiring to understand their environments more completely when they were used in other types of experiments, genetic and economic control especially, and in some cases appreciating possible value as subjects in general nutritional studies. The small amount of food required, short life cycles, and simple laboratory requirements for rearing have been attractive characteristics.

Of insects thus studied, the fruit fly, *Drosophila melanogaster*, has received most attention. This species normally lives upon decaying fruit but Delcourt and Guyenot (3) have reported a method of securing aseptic colonies reared upon potato and dead bakers' yeast while the potato alone produced few, stunted, and sterile adults. Guyenot (4) further found that they could reproduce upon yeast alone and a synthetic diet containing yeast or its alcoholic extract and suggested the similarity of the essential fraction of yeast to Funk's "vitamine." Loeb and Northrop (5) and Northrop (6) verified these findings regarding the value of yeast and its extract in diets for this insect. Baumberger (7) in an extensive study thought the essential portion of yeast was its nucleoprotein but the growth-promoting substance may have been present in his preparation. Glaser (8) thinks that fresh, uncontaminated, orange or grapefruit juice contains an essential substance destroyed by his method of sterilization. Most recently Bacot and Harden (9) using a purified basal diet consisting of caseinogen, starch, salts, and cane sugar, all extracted with alcohol, reared aseptic cultures of the flies when yeast or the alcoholic extract of wheat germ was added with butter fat and with or without fresh lemon juice. They were not certain whether the butter fat was necessary or not.

Other insects living upon decaying material whose nutritional requirements have been investigated are the blow flies studied by Bogdanow (10) and Wollman (11) with experiments inadequately planned for conclusive results.

Another type of insect, that infesting cereals and their products, from the nature of its food habits offers even greater possibilities for such experimental use. *Tenebrio molitor* was studied by Portier (12) who reared its larvæ as rapidly on a sterilized as on an unsterilized medium, thus demonstrating that the presence of microorganisms was unessential, and Passarini (13) who found a favorable influence of the pericarp of cereals. Chapman (14) has conducted extensive research with the confused flour beetle, *Tribolium confusum*, in which he used purified diets consisting of cornstarch, various proteins, salts, and wheat germ or the vitamin B-containing fraction. He concluded that the wheat germ itself was a more satisfactory

supplement to his purified basal rations than the vitamin B portion (an alcoholic extract on dextrin) but none of his basal rations contained fat. Evidence will be pointed out in this paper that fat considerably accelerates growth on an otherwise adequate diet. Furthermore, the single purified proteins used were often incomplete, hence the embryo may have helped cover an amino acid deficiency. Such considerations mean that the vitamin B content of the embryo was not demonstrated to be unessential and further evidence to this effect was afforded by the fact that only rarely did an adult emerge on a purified ration lacking the embryo or its extract as sources of this vitamin.

Richardson (15) studied the effect of the extraction of whole wheat with various solvents on the growth of *Ephestia kuehniella*. Since the removal of ether-soluble material retarded or prevented development and the addition of egg yolk extract or the return of the extracted portion permitted it, but olive oil and lard did not, he concluded that a vitamin A deficiency was indicated. It is possible that wheat contains a small amount of this vitamin as Steenbock and Coward (16) have pointed out, but the negative effects reported for butter are not adequately explained. One cannot accept Richardson's negative results in the face of his admission that they were sometimes different when he used purified solvents.[1] He derives a conclusion that vitamin B is necessary because alcoholic extraction hinders development but he reports no extraction drastic enough to prevent all transformations, nor does he say whether the moisture content of these extracted materials was adjusted or not; it is known that this insect is sensitive to this factor.

In general, it may be concluded that the work of Guyenot, Northrop, and Bacot and Harden seems to show that *Drosophila* requires vitamin B but there is no conclusive evidence that vitamin A is essential. The investigations of Chapman and Richardson indicate a need for vitamin B on the part of the two cereal-infesting insects studied but the conclusion cannot be unmistakably derived from the experiments as planned and reasons for doubting the conclusion of the latter, that vitamin A is necessary, have been stated.

EXPERIMENTAL.

Tribolium confusum is a very cosmopolitan member of the coleopterous family Tenebrionidæ. Adults and larvæ are common

[1] The writer carried on some experiments with *Ephestia* to parallel those here reported for *Tribolium* but results with the former were not so complete because of decimation of many cultures by disease. However, adult moths have been produced on the wheat embryo extracted with ether as later described in this paper and supplemented with Crisco, a fat lacking vitamin A. Furthermore, the ether-alcohol-extracted embryo of the same series produced a 50 per cent yield of moths in one culture of twenty when supplemented by the alcoholic extract and Crisco.

pests of cereals and allied products. At a constant temperature of 28° and relative humidity of 70 per cent the egg stage lasts about 6 days, the larval period on whole wheat about 21 days, and the pupal period 7 days. These stages can be shortened by raising the temperature and in some of these experiments this was done. The larval period is the period of growth and the adequacy of a ration can be measured by the number of days required from hatching to pupation when other conditions are constant. The full grown larvæ are about $\frac{1}{4}$ inch long.

Technique of Handling Tribolium.—A large number of adults are transferred to patent flour that has been sifted with fine bolting cloth (No. 5 XX silk) to remove particles which would otherwise appear with the eggs. The container is kept in a warm place (room temperature or incubator up to 31°) for a day or two and then the beetles can be removed with a coarse sieve. Resifting with the bolting cloth removes the eggs which are readily recognizable as tiny pellets, being covered with a coating of flour which adheres to them upon laying. These are placed in an incubator and upon the day of hatching are transferred by means of a camel's-hair brush to the vials containing the experimental rations. 24×90 mm. homeopathic vials containing approximately 5 gm. of food are adequate for at least twenty larvæ. These containers are plugged with absorbent cotton and retained in an incubator until pupation occurs. Duplicate vials are prepared for each test.

In the present experiments we used in some cases a Carrier cabinet maintaining a relative humidity of 70 per cent by the spray process; in others a constant temperature incubator in which the bottom was covered with a pan of saturated NaCl solution which maintains a relative humidity of about 75 per cent was found to be satisfactory. This difference in relative humidity did not seem to be significant. Constant temperature facilitates comparison of results run at different times or differing in the length of time required until pupation. As the time for pupation approached the cultures were examined daily by emptying the contents of the vial onto a sheet of paper. Transformations could thus be easily observed, recorded, and pupæ removed. The remaining larvæ were poured with the food back into the vial.

The moisture content of all of the rations was made up to approximately 10 per cent but this is unnecessary if the foodstuffs

are held at the above relative humidity for 2 or 3 weeks before larvæ are added since an equilibrium takes place which gives an adequate moisture content.

Analysis of Nutritional Requirements of Tribolium.

Experiment 1. Effect of Ether and Alcohol Extraction of Wheat Embryo upon Growth.—Commercial wheat embryo was found to be an optimum food for this insect and hence was made the basis of a

TABLE I.

Growth of Tribolium on Commercial Wheat Germ and after Extraction with Alcohol and Ether.

Temperature, 28°; relative humidity, 70 per cent; twenty larvæ per vial.

Ration No.	Ration content.	Days in larval period.		No. of pupæ.
		Mean.	C.V.*	
	per cent			
1 A	Commercial wheat embryo.	21.2 ± 0.094	2.94	20
		20.8 ± 0.108	3.22	19
25 A	Embryo extracted 3 wks. with alcohol and ether.			0†
				0
26 A	Ration 25 A..... 90			0†
	Butter fat..... 10			0
27 A	Ration 25 A..... 90			0†
	Crisco..... 10			0
28 A	Ration 25 A + alcoholic extract = 10 per cent embryo.	30.6 ± 0.384	8.10	19
		28.9 ± 0.305	6.59	20
29 A	Ration 28 A..... 90	24.1 ± 0.384	5.59	19
	Butter fat..... 10	23.0 ± 0.146	4.05	20
30 A	Ration 28 A..... 90	26.0 ± 0.298	7.45	20
	Crisco..... 10	25.3 ± 0.238	6.22	20

* Coefficient of variability.

† The same results when repeated.

series of experiments to determine as definitely as possible the essential constituents for growth. The embryo as purchased was extracted with ether 48 hours, then with cold 90 per cent alcohol, followed by the same hot to a total extraction period of 3 weeks, all in a Lloyd continuous extractor. In making up the experimental rations fat was returned to the amount of 10 per cent,

slightly less than the ether extract obtained. The alcoholic extract was evaporated onto dextrin and used as an aliquot of the original. Butter fat was prepared for this work by rendering and filtering to remove the curd and water. Results are given in Table I.

Since all fat and fat-soluble substances must have been removed from the embryo by this extended extraction it is evident that neither fat nor fat-soluble vitamins are absolutely limiting growth

TABLE II.

Growth of Tribolium on Ration of Purified Constituents with and without Vitamins A and B.

Temperature, 28°; relative humidity, 70 per cent; twenty larvæ per vial.

Ration No.	Ration content.		Days in larval period.		No. of pupæ.
			Mean.	C.V.*	
		per cent			
1 B	Casein	28			
	Osborne-Mendel salts	4			0
	Dextrin	68			0
2 B	Casein	28			
	Osborne-Mendel salts	4			
	Dextrin	58			0
	Butter fat	10			0
3 B	2 B with Crisco in place of butter fat.				0
					0
5 B	Casein	28			
	Osborne-Mendel salts	4			
	Alcoholic extract = germ 10 per cent		28.4 ± 0.340	7.75	19
	Dextrin to	100	29.1 ± 0.340	7.56	19
6 B	5 B with 10 per cent butter fat in place of dextrin	10	26.4 ± 0.408	10.00	19
			26.8 ± 0.438	10.34	18
7 B	5 B with 10 per cent Crisco in place of dextrin	10	25.5 ± 0.349	8.59	18
			26.1 ± 0.238	5.90	19

* Coefficient of variability.

factors. On the other hand, the fact that a return of a 10 per cent equivalent of the alcoholic fraction permits pupation indicates a need for vitamin B. The retardation noted on comparison of this ration (No. 28 A) with the control was almost overcome by the addition of a fat, the presence of vitamin A seeming to be insignificant.

Experiment 2. Growth on Ration of Purified Constituents with and without Vitamins A and B.—Since commercial wheat embryo was taken as the control ration for the above experiment a purified foods series was planned with a basal ration approximating the embryo in composition to study the effect of the presence or absence of the two vitamins, A and B. This basal ration contained casein 28, Osborne-Mendel (17) salts 4, fat 10, and dextrin to 100.

Dextrin was prepared from commercial corn-starch by moistening with 0.2 per cent citric acid and autoclaving 5 hours at 15 to 20 pounds pressure. The dried product was ground and extracted with cold 90 per cent alcohol 24 hours, the same hot 48 hours, and ether 36 hours, all in a Lloyd continuous extractor. Casein was prepared as described by Palmer and Kennedy (18).

When yeast is mentioned, reference is to the pure, dried, product of the Northwestern Yeast Company. A summary of the results on this series of rations is given in Table II.

The similarity (compare Tables I and II) between the pairs, Rations 28 A and 5 B, Rations 29 A and 6 B, and Rations 30 A and 7 B, is so remarkably exact that the same observations apply. Thus it is seen that a purified diet can be prepared for this insect, which gives practically the same growth rate as the equivalent rations prepared from extracted wheat embryo. In the data here presented the growth rate in neither case quite attained that on the untreated embryo.

Applications in Vitamin B Studies.

Experiment 3. Quantitative Studies of the Effect of Additions of Sources of Vitamin B to a Purified Basal Ration.—Since these insects seemed to be sensitive to the presence or absence of vitamin B in their food materials an attempt was made to determine whether their growth rate might not be a means of measuring that constituent. The basal ration consisted of casein 28, Osborne-Mendel salts 4, ether extract of wheat germ 3, and dextrin to 100. This percentage of fat was used because of evidence that certain irregular results might be due to a slight excess or failure to incorporate it into the mixture so that there was no greasiness. 3 per cent of fat is approximately the proportion found in whole wheat, another optimum food material. The proportions of

sources of vitamin B are plotted against days in the larval period in Fig. 1.

It is evident from the curves that result that this insect responds in a quantitative fashion to amounts of growth-promoting substance over a certain range of proportions. Response was some-

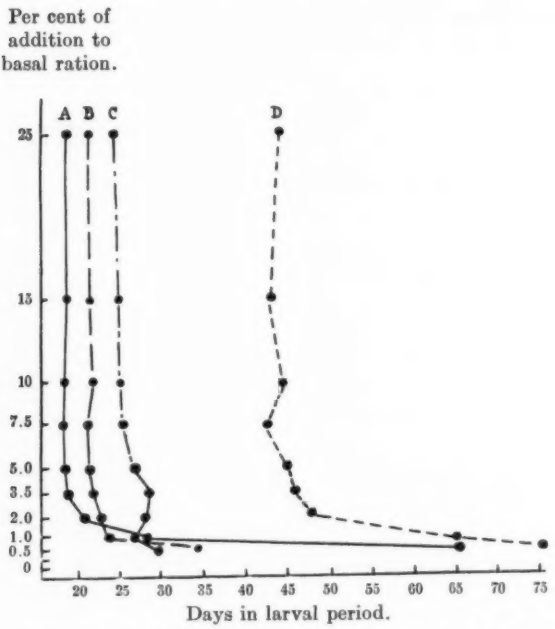

FIG. 1. Growth of *Tribolium* when a source of vitamin B is added to a purified basal ration in varying proportions. Addition to basal ration: *A*, wheat embryo; *B*, alcoholic extract equivalent to wheat embryo; *C*, yeast; *D*, alcoholic extract equivalent to yeast. Basal ration: casein 28, Osborne-Mendel salts 4, ether extract of wheat embryo 3, dextrin 65.

what irregular at the lower levels but this may be partially due to weaknesses in the technique of preparation of the rations. It is probably difficult to secure a uniform distribution of a 1 per cent fraction of such a mixture with a mortar and pestle. All of the ingredients for this series were sifted through fine bolting cloth

before mixing and for such refined work no doubt this should have been repeated after combination. Sherman and Spohn (19) say that by feeding their basal diets "to experimental animals of suitable age and size and sufficiently matched as to nutritional history and litter controls, it is believed to be possible, when dealing with averages of ten or more rats on each diet, to detect a diminution certainly of 25 per cent and probably of 15 per cent in the vitamin B content of the food tested." In each case there is a definite decrease in growth rate of the insects when the source of vitamin B is diminished from 2 to 0.5 per cent of the ration and on some curves more sensitivity is indicated by the variation over a wider range. It is also to be noted that *Tribolium* is remarkably sensitive to very small amounts of vitamin B, as little as 0.5 per cent of a source being sufficient to produce some pupæ.

Another condition lessening exactness is the greater scatter of the individual cases, resulting in a high probable error of the mean at the low levels, a state found on all rations which are far from optimum. In this connection, it is interesting to find that Sherman and Spohn (19) report for rats that "the coefficient of variation is greatest for the diet containing (of those compared) the smallest amount of vitamin and becomes less as the vitamin intake approaches the optimum." Individual variations were larger on diets permitting only subnormal growth and as these were brought nearer optimum the rate of growth became both more rapid and more uniform.

But an even more outstanding result of this series was the rather unexpected qualitative differences indicated among the four sources of vitamin B which showed no tendency to diminish at high levels of feeding.[2] These differences correlate with the previous failures to secure quite optimum growth on a purified ration supplemented either with yeast or the alcoholic extract of wheat embryo. Wheat embryo seems to contain some growth-promoting substance absent from its alcoholic extract or yeast. This phase of the problem is being investigated further for its relationship to the question of the multiplicity of factors involved in the vitamin B complex. Evidence on this point has been con-

[2] The extract of yeast was prepared by Dr. Cornelia Kennedy by extraction in a Soxhlet extractor with 85 per cent alcohol until the extract came through colorless.

stantly accumulating and has been most recently summarized by Sherman and Axtmayer (20).

Experiment 4. Use of Tribolium in Following the Chemical Purification of Vitamin B.—This experiment had a twofold purpose, an attempt to identify more closely the growth-promoting

TABLE III.

Purification of Vitamin B Fraction of Brewers' Yeast According to Methods of Osborne and Wakeman, and of Levene and van der Hoeven and the Growth-Promoting Power of Product and Residue at Various Stages.

Temperature, 31–32°; relative humidity, 75 per cent; twenty larvæ per vial.

Ration No.	Addition to basal ration.		Days in larval period.		No. of pupæ.
			Mean.	C.V.*	
		per cent			
14 B	Dried brewers' yeast.....	10	16.3 ± 0.865	3.52	20
			16.3 ± 0.708	2.87	20
15 B	" " "	1	16.9 ± 0.081	3.18	20
			16.8 ± 0.077	2.83	20
16 B	Osborne-Wakeman fraction on dextrin (roughly equal to 30 per cent dried brewers' yeast)...	10	30.1 ± 0.294	5.78	16
			31.8 ± 0.591	12.42	16
17 B	" "	1	30.4 ± 0.421	7.41	13
			32.2 ± 0.729	12.53	14
18 B	Product of the first release at pH 3 dried on dextrin†.....	10	34.5 ± 0.512	9.03	17
			34.1 ± 0.684	11.90	16
19 B	" "	1	32.5 ± 0.729	12.85	15
			34.6 ± 0.493	8.97	18
20 B	Final Levene-van der Hoeven product.‡.....	2	32.0 ± 1.37	16.85	7
			31.2 ± 1.37	14.52	5
21 B	" "	1	32.8 ± 0.762	11.05	10
			31.2 ± 0.644	9.22	9
22 B	" "	0.1	37.0 ± 1.32	14.89	8
			39.3 ± 1.22	14.55	10
23 B	Filtrate after adsorption dried on 10 gm. dextrin (see note to Ration 18 B).....	10	30.0 ± 0.561	10.36	14
			30.7 ± 0.923	16.10	13
24 B	" "	1	30.8 ± 0.758	14.00	15
			30.3 ± 0.543	11.24	18

TABLE III—*Concluded.*

Ration No.	Addition to basal ration.	Days in larval period.		No. of pupæ.
		Mean.	C.V.*	
	per cent			
25 B	Final acetone filtrate dried on 10 gm. dextrin (see note to Ration 20 B)..... 10			2§ 0
26 B	" " 1			5§ 2

* Coefficient of variability.

† The Osborne-Wakeman fraction from approximately 170 gm. of dried brewers' yeast was deaminized by NO_2 and the solution subjected to adsorption with silica gel at pH 5. A first release was made at pH 3 and this solution dried on 10 gm. of dextrin. This portion was thus kept separate from later releases to insure greater purity from material carried down mechanically.

‡ The product of a second release when the silica gel adsorption combination was returned to pH 3 after neutralization plus that obtained at pH 9.5 was concentrated and precipitated by dry acetone.

§ Cultures discontinued on 68th day with some larvæ remaining which might have pupated.

substance found in alcoholic extracts by preparing one of the most purified forms of vitamin B reported in the literature, and an examination of the usefulness of *Tribolium* in following results at each stage in a chemical procedure.

The recent method of Levene and van der Hoeven (21) was followed. This is based upon the discovery that the vitamin B fraction of yeast can be precipitated from water and dilute alcohol solutions by bringing the alcoholic content to about 79 per cent (Osborne and Wakeman (22)) and that further concentration can be secured by adsorption on silica gel at pH 5 and release by increasing acidity to pH 3 or alkalinity to pH 9.5. In these experiments a basal ration was used that consisted of casein 28, Osborne-Mendel salts 4, Crisco 3, and dextrin 65. To this were added definite amounts of the concentrated material as it occurred at different stages in the process. The residue as well was tested. The cultures were reared in an incubator with little variation from 31° in temperature and 75 per cent relative humidity. These

conditions produced pupation in the control wheat germ in 14 to 15 days, a much accelerated rate over that at 28°. Results are summarized in Table III.

Further evidence is offered by this work that water or alcoholic extracts of yeast are never equal in growth-promoting power to the yeast itself. The time for pupation is doubled and the yield reduced. The presence of the necessary substance in every fraction as this process was developed indicates the source of at least some of the losses reported by Levene and van der Hoeven. It is to be noted that these insects are very well adapted to testing such a chemical purification process because they can detect so

TABLE IV.

Growth of Tribolium on Different Fractions of Wheat Kernel (Triticum vulgare).

Temperature, 28°; relative humidity, 70 per cent; twenty larvæ per vial.

Ration No.	Ration content.	Days in larval period.		No. of pupæ.
		Mean.	C.V.*	
1 C	Ground whole kernel.	21.7 ± 0.252	7.49	20
2 C	Wheat embryo removed by hand.	21.2 ± 0.119	3.76	20
3 C	Half of kernel near embryo end, embryo removed.	21.7 ± 0.084	2.57	20
4 C	Remainder of kernel.	25.4 ± 0.188	4.79	19
5 C	Patent flour.	27.3 ± 0.455	10.10	17
		28.3 ± 0.595	13.94	20

* Coefficient of variability.

noticeably and quickly such small amounts of the growth-promoting material. The fact that the final acetone filtrate was apparently much less abundantly supplied with the vitamin indicates that it is a good precipitant for that factor.

0.1 per cent, 1 part in 1000, of the final product did produce pupæ but the extent of the concentration cannot be judged because the supplementary power of the yeast itself in such a small proportion was not tested. When the experiments were planned it was expected that the 1 per cent level would be limiting but it was not (see Ration 15 B).

Experiment 5. Adequacy of Portions of Fractionated Cereals for Growth of Tribolium.—One phase of the nutrition of this cereal-

infesting insect which it was thought might throw some more light upon the requirements of the insect itself as well as furnish data for comparison with what is known of the adequacy of these food

TABLE V.

Growth of Tribolium on Different Fractions of Corn Kernel (Zea mays).

Temperature, 30°; relative humidity, 75 per cent; twenty larvæ per vial.

Ration No.	Ration content.	Days in larval period.		No. of pupæ.
		Mean.	C.V.*	
6 C	Whole yellow corn (Minnesota 13).	29.7 ± 0.691	15.45	20
7 C	Whole white corn (Silver King).	24.6 ± 0.336	8.54	18
8 C	Embryo yellow corn.	27.5 ± 0.746	17.52	19
9 C	" white "	26.1 ± 0.702	17.40	19
10 C	Remainder of kernel, embryo removed, yellow.	29.8 ± 0.417	8.94	19
11 C	Remainder of kernel, embryo removed, white.	26.0 ± 0.270	6.58	20

*Coefficient of variability.

TABLE VI.

Growth of Tribolium on Different Fractions of Barley (Hordeum vulgare).

Temperature, 31–32°; relative humidity, 75 per cent; ten larvæ per vial.

Ration No.	Ration content.	Days in larval period.		No. of pupæ.
		Mean.	C.V.*	
12 C	Whole barley kernel (hull removed).	14.6 ± 0.104	3.35	10
		14.6 ± 0.104	3.40	10
13 C	Barley embryo.	14.5 ± 0.106	3.45	10
14 C	Embryo end of kernel, embryo removed.	14.6 ± 0.093	3.03	10
		14.6 ± 0.093	3.03	10
15 C	Remainder of kernel.	18.7 ± 0.242	5.57	9
		19.0 ± 0.165	4.07	10

* Coefficient of variability.

materials from research conducted with other animals, is a study of its growth on different cereals and their fractions. All of these fractions were carefully prepared by hand dissection with a sharp

dental scalpel and grinding with a mortar and pestle. The part of the experiment which deals with wheat was run at the usual temperature of 28° and 70 per cent relative humidity, that with

TABLE VII.

Growth of Tribolium on Different Fractions of Oats (Avena sativa).

Temperature, 31–32°; relative humidity, 75 per cent; ten larvæ per vial.

Ration No.	Ration content.	Days in larval period.		No. of pupæ.
		Mean.	C.V.*	
16 C	Whole oat kernel (hull removed).	16.6 ± 0.190	3.40	10
		16.9 ± 0.177	4.91	10
17 C	Oat embryo.	16.4 ± 0.195	5.54	10
18 C	Embryo end of kernel, embryo removed.	16.2 ± 0.159	4.61	10
		15.9 ± 0.212	5.59	9
19 C	Remainder of kernel.	16.8 ± 0.314	8.31	9
		16.8 ± 0.314	8.31	9

* Coefficient of variability.

TABLE VIII.

Growth of Tribolium on Different Fractions of Rice Kernel (Oryza sativa).

Temperature, 31–32°; relative humidity, 75 per cent; ten larvæ per vial.

Ration No.	Ration content.	Days in larval period.		No. of pupæ.
		Mean.	C.V.*	
20 C	Whole rice kernel (hull removed).	17.0 ± 0.135	3.71	10
		17.2 ± 0.085	4.32	10
21 C	Polished rice.	21.8 ± 0.368	4.43	3
		27.0 ± 1.770	25.61	7
22 C	Embryo end of polished rice with embryo completely removed.	31.2 ± 2.47	23.45	4
		40.6 ± 3.22	26.24	5
23 C	Remainder of kernel.	47.0 ± 2.31	16.90	5
		39.7 ± 3.64	27.20	4

* Coefficient of variability.

corn at 30° and 75 per cent relative humidity, and the remainder at 31–32°. Results are given in Tables IV to VIII inclusive.

It is of course evident that this type of experiment does not permit determination of the identity of a limiting factor. All one

can say is that some more or less quantitative or qualitative deficiency is indicated by comparison of results but even this might have a certain significance in the case of cereals completely ana-

TABLE IX.

Growth-Promoting Power of Wheat Kernel and Its Fractions When Added to Purified Ration Lacking Vitamin B.

Temperature, 31–32°; relative humidity, 75 per cent; twenty larvae per vial.

Ration No.	Addition to basal ration.	Days in larval period.		No. of pupæ.
		Mean.	C.V.*	
	per cent			
24 C	Whole wheat..... 20	17.0 ± 0.201	7.42	18
		16.4 ± 0.161	6.32	19
25 C	" " 10	18.1 ± 0.299	10.95	20
		19.1 ± 0.322	11.23	19
26 C	" " 1	39.2 ± 3.75	31.62	5
		46.7 ± 3.03	27.10	8
27 C	Dissected wheat embryo... 10	16.5 ± 0.178	6.62	17
		16.8 ± 0.185	6.59	18
28 C	" " " ... 1	20.2 ± 0.226	7.81	20
		20.6 ± 0.293	9.44	20
29 C	Embryo end of kernel, embryo removed..... 20	16.2 ± 0.178	7.28	20
		(Duplicate omitted by mistake.)		
30 C	" " 10	19.5 ± 0.283	9.61	20
		17.6 ± 0.138	7.84	20
31 C	" " 1	47.4 ± 3.58	29.55	7
		45.0 ± 2.56	21.65	6
32 C	Remainder of kernel..... 20	23.6 ± 0.395	11.10	20
		23.4 ± 0.447	12.64	20
33 C	" " " 10	30.7 ± 1.145	19.10	12
		(Duplicate omitted by mistake.)		
34 C	" " " 1	36.0 ± 2.02	25.0	2
		48.0 ± 2.23	11.38	3

* Coefficient of variability.

lyzed biologically in the case of mammals. Such applications will be pointed out later.

In the case of wheat some factor in the endosperm is at a growth-limiting level, but whole wheat, the embryo, or the embryo end

of the kernel gives uniformly normal growth (Table IV). In the corn experiment white and yellow varieties were compared with no result indicating a superiority in the yellow due to its vitamin A content. This agrees with previous evidence of absence of sensitivity to this factor. Croll and Mendel (23) reported a lack of vitamin B in corn endosperm but removal of the embryo did not consistently prolong the growth of *Tribolium* (Table V). Barley (Table VI) gave normal growth if the embryo or embryo end of the endosperm was present but the distal end of the kernel produced

TABLE X.

Growth-Promoting Power of Corn Kernel and Its Fractions Added to Purified Basal Ration Lacking Vitamin B (Yellow Corn).

Temperature, 31–32°; relative humidity, 75 per cent; twenty larvæ per vial.

Ration No.	Addition to basal ration.		Days in larval period.		No. of pupæ.
			Mean.	C.V.*	
		per cent			
35 C	Whole corn	20	21.4 ± 0.218	6.45	19
36 C	" "	10	21.5 ± 0.188	5.02	15
37 C	" "	1	34.7 ± 1.38	17.75	9
38 C	Corn embryo	20	21.0 ± 0.289	8.92	19
39 C	" "	10	23.1 ± 0.331	9.58	17
40 C	" "	1	26.7 ± 0.437	10.00	17
41 C	Remainder of kernel	20	21.6 ± 0.154	4.27	19
42 C	" " "	10	26.5 ± 0.443	9.28	14
43 C	" " "	1	38.6 ± 1.37	14.94	8

* Coefficient of variability.

retarded development when used as the entire ration. This is very similar to the results with wheat. The fractions of the oat kernel (Table VII) were essentially similar in effect but none was the equal of whole wheat or barley. Whole rice (Table VIII) is very like oats but it is remarkable that polished rice or the endosperm alone permitted a fair percentage of pupations in spite of the belief that this portion entirely lacks vitamin B (McCollum and Davis (24)). This is probably evidence that these animals can exist on a lower level of this factor than the usual laboratory animals, possibly because their growth period is more elastic.

One is led to speculate on the probability of the presence of this vitamin in all once living structures.

Experiment 6. Use of Tribolium for Determination of Vitamin B in Portions of Cereals Added to a Purified Basal Ration Lacking That Factor.—Bell and Mendel (25) and Croll and Mendel (23) have made detailed studies of the vitamin B content of wheat and corn and their fractions using rats and mice as experimental animals. Since previous results with *Tribolium* indicated a sensitivity to this vitamin a group of rations was planned so that results might be comparable. The basal ration and conditions of the experiment were again those of Experiment 4.

The rations and larval periods for the wheat determinations are given in Table IX. The comparative abundance of the vitamin in the embryo is evident from comparative growth on Rations 26 C, 28 C, 31 C, and 34 C. Increase of endosperm distal to the embryo is associated with decrease of vitamin B. This conclusion is drawn from the great drop in growth rate when Rations 30 C and 33 C are compared. The small number of pupæ on Ration 34 C does not permit its comparison with Ration 31 C. This conclusive evidence that the embryo and its adjacent endosperm are richest in this vitamin duplicates the findings of Bell and Mendel in the work just mentioned.

For some undetermined reason corn does not supplement this basal ration so well as wheat (Table X). 10 per cent of whole corn is equal in growth-promoting power to 20 per cent but the rate is still 25 per cent below that with wheat. Results on the 1 per cent levels show a superiority for the germ in supplementary value but the remainder of the kernel is surprisingly good considering the results reported by Croll and Mendel. They state that practically all of the vitamin B in corn is in the embryo. It is possible that this contradiction involves the plurality of the vitamin B factor and in any case the growth-promoting difference demonstrated between wheat and corn is related to this question. Further research is needed to clear up this point.

DISCUSSION.

It has been suggested that investigations of the phylogenetic development of nutritional requirements might yield valuable implications in the understanding of needs of higher animals.

From this work it seems that *Tribolium confusum* has no absolute requirement for fat-soluble substances for growth and reproduction of one generation at least. (The adults emerging in different types of rations including one of purified constituents with the alcoholic extract of wheat germ and no fat were held for a time and the appearance of larvæ was later noted in all.) Sherman and Storms (26) concluded from results with rats that there was no good evidence of synthesis of vitamin A in the animal body at any age. It should be possible to rear these larvæ on a fat-free or vitamin A-free diet in sufficient numbers to determine their content of this substance. Culturing intestinal contents on several types of media failed to reveal any consistently occurring organism that might be a symbiotic source of nutritional constituents. When the larvæ or various types of rations were exposed to daily ultraviolet irradiation no effect was detectable. Evidently this species either possesses remarkable synthetic ability or the physiologic functions whose regulation in mammals is concerned with these vitamins have a different mechanism.

The vitamin B type of accessory substance was not only a necessary constituent of the ration of *Tribolium* but was present in the larval bodies in sufficient amount to produce a fair rate of growth in rats when 0.5 gm. was fed daily. Absence of vitamin C requirement is, of course, not peculiar to this species. In general such wide differences in vitamin requirements between animals of different phyla lend further weight to the view that these substances are so widely different in composition and function that their present classification under one name will in the future be discarded.

This work has especially indicated the utility of *Tribolium* in vitamin experiments but its ease of manipulation on purified diets and small food requirements point to possibilities in such other biological analyses as those of protein values.

CONCLUSIONS.

1. It is possible to determine the vitamin requirements of an insect, *Tribolium confusum*, a common pest in such human food materials as the cereals, by using purified or otherwise modified rations with essentially the procedure followed with the ordinary laboratory animals.

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2. This insect can be rapidly reared on a purified ration of protein, salts, dextrin, and a small fraction of yeast, wheat germ, and their alcoholic extracts, though the latter are not so completely supplementary as the former. The addition of a fat with or without vitamin A accelerates the growth rate.

3. The distribution of the necessary growth-promoting substance is similar to that of vitamin B as defined by mammalian growth behavior and it is at least in part identical with the purified products of the Osborne and Wakeman, and Levene and van der Hoeven purification processes. Some evidence that a plural substance is concerned is pointed out.

4. *Tribolium* is sensitive qualitatively to as low as 0.5 per cent of a source of this growth-promoting substance or substances and apparently makes a quantitative response to its additions over a certain range.

5. Growth on fractions of wheat, corn, oats, barley, and rice indicates no absolutely limiting factor in any fraction tried.

6. When wheat and its fractions are added to a purified basal ration lacking vitamin B the embryo and adjacent portion of the kernel are shown to be the most abundantly supplied with this factor. A similar study with corn demonstrated the presence of the necessary vitamin in the endosperm.

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THE DETERMINATION OF SMALL AMOUNTS OF LIPID IN BLOOD PLASMA.

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In 1914 (1) the author published a method for the determination of the lipids of blood or blood plasma, in which the nephelometric principle was made use of. The cholesterol and mixed fatty acids of the saponified sample were measured together, and the value for fatty acid was determined by subtracting from the total fat (fatty acids plus cholesterol) the value for cholesterol determined colorimetrically. Results were obtained which agreed quite well with those obtained by a standard macro method (Kumagawa-Suto (2)) but the procedure appeared theoretically faulty because the nephelometric coefficients of cholesterol and the fatty acids were quite far apart (3, 4). Consequently a modification of the method was made in which the fatty acids were determined separately (5). The same theoretical objection that the nephelometric values of the fatty acids present (mainly oleic and palmitic acids) were far apart was answered by the fact that the melting point of the lipid mixture as measured was a more important factor in the values obtained than the substances themselves, a mixture which gave a solid (suspension) colloid when suspended in water, behaving quite differently from a mixture which gave a liquid (emulsion) colloid. Consequently a standard was used consisting of a mixture of fatty acids having approximately the same melting point as the fatty acid mixture of blood which had been shown to be fairly constant for a number of animals. That the theoretical objections had no important practical bearing was sufficiently shown by the fact that the values obtained by both procedures agreed very well with those obtained by the commonly used macro methods and by at least one of the micro methods, facts which

should be emphasized since they have not been mentioned by the critics of the method. The reason why it has been possible to measure in one mixture substances of such different nephelometric properties as cholesterol, palmitic, and oleic acids was that it was their behavior as a mixture rather than as individuals which was involved. Nevertheless the success of the method appeared to depend to a considerable extent on the constancy of the blood fatty acid mixture, and, while this seemed to hold over a variety of conditions (6), it was not hard to imagine conditions, *e.g.* during the absorption of large amounts of fat from the intestine, when the mixture might change radically in composition and the procedure would be liable to error in so far as the new mixture differed in its nephelometric properties from that of the mixture present in the blood in the postabsorptive state. The nephelometric method therefore seemed to lack flexibility. Other objections to the procedure were that, as noted above, it is difficult to defend on theoretical grounds, at least in the light of present knowledge, and has the practical disadvantage of requiring a special instrument—the nephelometer—one which some workers apparently cannot use successfully. Moreover, the fatty acid suspension is not an easy one to work with, being relatively coarse, changing continuously in value, and requiring therefore a standard which changes in value at approximately the same rate. A procedure was sought which would be more independent not only of the lipid mixture of blood but also of the personal factor, and preferably one which called for ordinary quantitative apparatus.

In 1918 Bang (7) announced an oxidative method suitable for use with the amounts of fatty material in 100 mg. of blood, and which appeared to offer the needed independence of the lipid mixture of blood since the factors found were very nearly the same for the common fatty acids and cholesterol. Regarding this method Blix, who has made extensive use of it in his work in diabetes, has made the following statements (4): "The common adoption of the method would then soon have been expected . . . . On the contrary it appears that the number of investigations published in which this method has been applied is remarkably few. The supposition is then near at hand that the method has been tried in many laboratories but for some reason or other rejected. Various investigators report unsuccessful attempts to

obtain reliable results by the method and point to certain sources of error difficult to control." Blix further states that in papers on investigations in which the method has been applied very little comment is given on it in spite of the fact that the figures found sometimes showed very marked discrepancies from corresponding figures published by Bang. Modifications of the method have been recorded by Condorelli (8) and Vasilewska (9) whose procedure is essentially Bang's, adapted for use with 1 mg. of fatty acid. Von Szent-Györgi (10) has offered a modification of the procedure applied to cholesterol which is notable in that a more complete oxidation was attempted although the values obtained were still some distance from the values for complete oxidation (factor of 3.35 as compared with the theoretical 3.92). On the other hand the method is not lacking in enthusiastic supporters of whom Blix (4, 11) and Fleisch (12) are two outstanding examples. These workers report large numbers of determinations claiming an accuracy which, considering the minute amounts of material worked with (about 0.1 mg.), is little short of marvelous, but which, with the limitations noted below, have been to a considerable extent corroborated by work done by the writer. Other workers reporting favorably on the procedure are Gade (13), who however substituted a new method, and very recently von Fellenberg (14) who reviewed the use of chromic acid oxidation and applied what is essentially the Bang procedure to the oxidation of a number of substances. The use of chromic acid oxidation for quantitative purposes is, of course, not new and has recently been made the basis of a method for the micro determination of carbon by Nicloux (15).

A significant fact regarding Bang's method is that the proponents of the method do not agree as to the basic factor for calculating their values. For example, in the case of oleic acid Fleisch agrees with Bang in using the factor 2.50, while Blix uses 2.06 (our own work with the Bang procedure as noted below gave a value of 2.29). Vasilewska (9), adapting the method to use with about 10 times the amount of material employed by Bang, used a factor of 2.65. These facts indicate a fundamental defect in the Bang procedure, which has probably been one of the stumbling-blocks in the way of the wide use of the method. The acquaintance of the present writer with Bang's method dates from its

first appearance in 1918 when an unsuccessful attempt was made to use it. Attention was again directed to the method by the work of Blix on diabetic lipemia (11) in which results obtained by Bang's methods were claimed to be at variance with those obtained by the nephelometric methods (16). With R. G. Sinclair of this laboratory the oxidative procedure of Bang's method was again tested out, particular attention being paid to the directions given by Blix for its use. At first no consistent results could be obtained with it on the common fatty acids—oleic and palmitic. It was finally decided that the method of driving off the solvent by heating on a sand bath was at fault because of the difficulty of controlling the heating, so a bath of Wood's metal heated to 145° (the temperature at which the solvent could be boiled off in the 70 seconds recommended by Blix) was substituted, with the result that consistent values were obtained, but the factor for oleic acid found was 1.68 as compared with Blix's factor of 2.06 and Bang's original figure of 2.50. Fleisch also found the sand bath recommended by Bang and Blix an unsuitable means of heating and used a procedure of his own (12).

From this preliminary work it became evident that, for the removal of the solvent and subsequent oxidation, the test-tube was not a suitable vessel, and for the remainder of the work on the method 125 cc., flat bottomed Erlenmeyer flasks with glass stoppers were used. The material in solution in petroleum ether was transferred to these flasks, the solvent removed by heating in a current of nitrogen (later air), and the oxidation and titration of the excess dichromate carried out according to the directions of Blix by use of a micro burette. Series of results in excellent agreement with each other were obtained by this procedure, amounts of oleic and stearic acids and cholesterol of 0.2 mg. being measured with an average error of 3 per cent and a maximum error of about 6 per cent (see Table I, Experiments 10 to 12), thus indicating that the procedure had in it the possibilities claimed by Bang. Nevertheless, as noted above, the factors for the substances determined did not agree with those of either Blix or Bang. Thus Bang's factors were oleic acid 2.50, cholesterol 2.45. Blix's factors were oleic acid 2.09, cholesterol 2.47, 2.50, palmitic acid 2.14, stearic acid 2.27. Fleisch found Bang's factors correct. Our own averages obtained in series of ten or more de-

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terminations in good agreement with each other (see Table I, Experiments 10 to 12) were oleic acid 2.29, cholesterol 2.62, stearic acid 2.26. Since blood values would be reported in terms of these factors, the absolute values of blood lipids reported by different workers would differ from each other in the same proportion, although data obtained by each might be comparable among themselves and could probably be compared with each other provided the oxidative factor had been determined sufficiently carefully and conditions of oxidation kept constant.

The reason for the differences in the oxidation factor is not far to seek, since the oxidation of the fatty acids by Bang's procedure is incomplete (less than two-thirds). The end-point would therefore be subject to various influences such as environmental temperature and rate of cooling of the reaction mixture (Blix states that the temperature factor is unimportant), and the presence of foreign substances either oxidizable or acting as catalysts.

It might be possible to standardize the method to give useful results as long as the procedure was strictly adhered to but this standardization might apply to only one set of conditions or to only one laboratory. It has, therefore, seemed to the writer more worth while to attempt a radical modification, if possible one in which the oxidation would be complete and the values therefore absolute. Complete oxidation would retain the advantage of the Bang procedure (comparative independence of the nature of the fatty material) and be more independent of environmental conditions. The results of the attempt are recorded below, and, while theoretical oxidation values have not been entirely attained (perhaps cannot be), the results are within a few per cent of absolute values and therefore near enough for present practical purposes.

The end aimed at was complete oxidation according to the equation (for palmitic acid) $C_{16}H_{32}O_2 + 23\ O_2 = 16\ CO_2 + 16\ H_2O$, from which it may be calculated that 1 mg. of palmitic acid should be completely oxidized by 3.59 cc. of 0.1 N dichromate when used with sulfuric acid, according to the equation $K_2Cr_2O_7 + 4\ H_2SO_4 = K_2SO_4 + Cr_2(SO_4)_3 + 4\ H_2O + 3\ O$. For oleic acid the factor would be 3.61, for stearic acid 3.66, and for cholesterol 3.92. It is notable that the three commonest fatty acids have thus theoretical combustion values very nearly the same (the greatest

difference between them being less than 3 per cent), while even for cholesterol the value is less than 10 per cent different. Since much of the reported difficulty with Bang's procedure could be traced to the effect of impurities on the minute amounts of material measured, the procedure was arranged for use with a larger amount, the basic weight chosen being 2 mg. By the use of this amount the effects of traces of impurities in reagents or slight accidental contamination was largely eliminated. Also by this means it was possible to avoid unusual apparatus, the whole determination being carried out with standard quantitative equipment. The general plan of the determination was to heat the fatty substance with an excess of sulfuric acid-dichromate oxidizing agent and to determine the amount of reagent used and so the amount of material oxidized by a titration of the unused reagent, use being made of the familiar iodometric procedure with standard thiosulfate. At first the plain sulfuric acid-dichromate reagent was used and it was demonstrated that excellent results could be attained with it (see Table I, Experiments 7 to 9), but the reagent plus the silver catalyst as introduced by Simon and Nicloux (15) was found to be a considerable improvement over the plain reagent in shortening the time of heating and increasing the certainty of the determination, so that it was finally adopted.

Attaining complete oxidation under conditions such that a quantitative measurement by difference was possible, was found to consist largely in adjustment of time and temperature of heating. The oxidizing mixture if heated high enough gives off its oxygen spontaneously to the air. The temperature must be so adjusted that this decomposition is negligible during the time of heating employed and yet that the reagent is in so sensitive a condition that the last traces of material are completely oxidized. The temperature of greatest sensitivity with least decomposition was found to be in the neighborhood of 124° and both temperature and time of heating were fairly critical. Not more than 2° above or below 124° was allowable, although 5 minutes longer time than that specified made only a negligible difference. The time of heating at this temperature with use of the plain reagent was found to be 25 to 20 minutes; with the silver reagent of Nicloux, 15 to 20 minutes. For the heating, ordinary electrically heated hot air ovens with temperature adjustment accurate to ± 2°

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were found sufficiently good. In ovens of this kind the temperature varies considerably from point to point and precautions must be taken to make sure that the temperature is the same in the samples, the control, and the vessel in which the thermometer is placed. To insure the even distribution of the heat, the ovens were fitted with a thick ($\frac{3}{8}$ inch) iron plate resting on a shelf in the oven and on which the flasks were set for heating. The thermometer was placed in a flask similar to those used for samples and

TABLE I.

Oxidation of Lipids.

Experiment No.	Kind and weight of material.	Reagent.	Time of heating.	Temperature.	No. of determinations.	Average 0.1 N dichromate per mg.	Variation from theoretical values.	Average variation from mean value.	Greatest variation from mean value.
			min.	*°C.*		*cc.*	*per cent*	*per cent*	*per cent*
1	Oleic acid, 2 mg.	Silver.	15	124	10	3.58	0.6	1.4	3.7
2	Palmitic acid, 2 mg.	"	15	124	31	3.46	3.08	1.0	3.0
3	Cholesterol, 2 mg.	"	15	124	20	3.81	1.30	2.0	5.2
4	Palmitic acid, 2 mg.	"	60	90	18	3.47	2.81	1.7	3.4
5	" " 2 "	"	60	89	16	3.50	2.00	1.7	4.4
6	Cholesterol, 2.4 "	"	60	89	20	4.58	2.00	1.0	2.5
7	Palmitic acid, 2 "	Plain.	25	124	18	3.57	0.0	1.04	3.0
8	" " 2 "	"	30	122	15	3.54	1.0	2.3	5.0
9	Oleic acid, 2 "	"	25	124	9	3.52	2.4	1.04	2.6
10	" " 0.2 mg.	"	20	22–24	16	2.29	36.6	1.2	1.2
11	Cholesterol, 0.2 mg.	"	20	22	12	2.62	35.4	2.2	6.0
12	Stearic acid, 0.2 "	"	20	22	16	2.26	38.4	2.0	4.7
13	Mixed palmitic acid and cholesterol, 2 mg.	Silver.	20	22	8	2.96	21.0	1.6	3.0

containing the same amount of reagent, the end extending through a hole in the top of the oven, so that readings could be made without opening the oven.

Equally good results were also obtained by conducting the heating on a water or steam bath at 88–90°, the time of heating being increased to 1 hour; in fact, better duplicates were ordinarily obtained by this procedure and the time of heating could be extended to 1½ hours without changing the values.

While determinations have been carried out in this laboratory under a considerable variety of conditions and it is believed that the times and temperatures stated will give the results claimed, it is perhaps too much to expect that similar conditions will always be found, and therefore small alterations of temperature and time of heating may have to be made.

The details of the method as used for the preliminary study of the oxidation of the fatty acids and cholesterol are given below in connection with its application to blood plasma. The results of typical series of consecutive determinations made by this procedure on known material are collected in Table I. The

TABLE II.

Oxidation of Varying Amounts of Lipids.

Results are expressed in cc. of 0.1 N dichromate per mg.

Lipid used.	1 mg. sample.	2 mg. sample.	3 mg. sample.	4 mg. sample.
Palmitic acid (3.57)*	3.62	3.53	3.52	3.54
	3.60	3.52	3.51	3.52
Cholesterol (3.92)	3.82	3.94		3.61
	3.90			3.81
	3.82			3.81
Cholesterol and palmitic acid, equal parts (3.74).	3.84	3.75	3.75	3.81
	3.85	3.75	3.78	3.75
	3.85	3.75	3.82	3.81
	3.73	3.74	3.70	3.75
	3.80	3.80	3.70	3.65

* The figures in parentheses indicate theoretical values.

substances used were oleic acid, Kahlbaum's K grade with an iodine absorption value of 90.0; stearic acid, Eastman Kodak Company, twice recrystallized from alcohol, m.p. 68.9°; palmitic acid, Eastman Kodak Company, m.p. 62°; cholesterol from human gallstones, recrystallized from alcohol to constant melting point at 145°. For the measurements these substances were dissolved in petroleum ether so that 1 cc. of solution contained 1 mg. of material.

Excess of Reagent.—To be certain of complete oxidation in the time allowed a large excess of reagent—more than double the theoretical amount—must be present. This is allowed for in the

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directions. That the reagent as used is adequate for oxidation over a considerable range of values is shown by the results in Table II. Amounts of from 1 to 4 mg. of fatty acids when determined according to the directions gave the values shown in Table II. Theoretical oxidation values are printed in parentheses.

Application to Plasma.

The oxidative procedure has been applied to the alcohol-ether extract of blood plasma as follows:

Reagents and Apparatus Required.—0.1 N sodium thiosulfate; N potassium dichromate; 1 per cent starch solution; 10 per cent potassium iodide.

Sulfuric Acid Reagent.—Pure concentrated sulfuric acid containing silver dichromate (silver reagent) prepared after Nicloux (15) as follows: To 5 gm. of silver nitrate dissolved in 25 cc. of water in a 100 cc. centrifuge tube were added 5 gm. of potassium dichromate dissolved in about 50 cc. of water. The precipitated silver dichromate was separated by centrifugation, washed twice by centrifugation with water to get rid of the nitric acid, and the cake of precipitate dissolved without drying in 500 cc. of pure concentrated sulfuric acid.

Petroleum Ether.—Commercial petroleum ether was fractionally distilled with a Clarke's (17) column, that which distilled above 60° being rejected. The distillate below 60° was washed with concentrated sulfuric acid and redistilled with an ordinary condenser. 50 cc. portions of this purified petroleum ether gave no blank when carried through the procedure below. Fair results can be obtained with unpurified commercial petroleum ether provided sufficient care be taken to remove the last traces of solvent from the fatty substances before oxidation (a difficult operation) and adequate control determinations made, but the purified material is well worth the effort of preparing it.

Alcohol-Ether Mixture.—3 parts of 95 per cent alcohol to 1 part of ordinary ether, both of which must be redistilled to remove traces of non-volatile organic substances.

Sodium Ethylate.—(Approximately N.) Made by dissolving 2 to 3 gm. of cleaned metallic sodium in 100 cc. of absolute alcohol, the solution being kept cool during the process. This reagent should be kept cool and in the dark and discarded when it becomes much colored.

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For the oxidation glass-stopped, Erlenmeyer flasks of 125 to 150 cc. capacity are required. These are cleaned for use by treatment with cleaning mixture (concentrated sulfuric acid and dichromate) for 1 hour in the hot air oven at 124°, then rinsed well with water, dried with heat, and kept stoppered.

Heating Apparatus.—(*a*) An electrically heated hot air oven with temperature adjustment accurate to ± 2°. The ordinary inexpensive hot air ovens of several makes were found satisfactory. These were fitted with a special shelf consisting of a $\frac{3}{8}$ inch iron plate. The ovens should be heated for at least 2 hours before use in order to come to constant temperature, and the temperature should be checked before each set of determinations.

(*b*) A water or steam bath which will maintain a temperature of 88–90°. The holes in the bath in which the flasks rest should be large enough just to hold the flask without allowing it to slip through.

Temperature Control.—The temperature in each case is to be measured by the use of a thermometer suspended in the liquid in a flask similar to the ones used for the determinations, the liquid being a measured sample of the mixture as used in the oxidations. The thermometer should be compared with a standard thermometer at the temperatures used. Since the time-temperature factor is critical, these details should be closely checked.

Extraction.—3 cc. of blood plasma were added to about 40 cc. of the alcohol-ether mixture in a 50 cc. volumetric flask. The plasma was run into the solvent in a slow stream and the flask was rotated during the process so that a finely flocculent precipitate of the protein was obtained. The flask was then immersed in boiling water with continuous rotation until the liquid boiled. It was kept at the boiling temperature for a few seconds, after which the liquid was allowed to cool to room temperature, made up to volume with alcohol-ether mixture, and filtered through a fat-free filter. In order to obtain the largest amount of filtrate possible the filter paper containing the protein precipitate was folded in the funnel and pressed out with a glass rod. The extraction of lipids from plasma by this procedure is practically complete as is shown by the fact that 25 cc. portions of plasma when treated in this way, filtered, the precipitate well washed

with alcohol-ether mixture, then digested with 20 per cent alkali for 8 hours, acidified, and extracted with ether, give no weighable residue. The statement appearing in the literature and attributed to the author that the alcohol-ether extraction is not complete by this procedure applies only to whole blood or corpuscles and not to plasma.

Determination.

Total Lipid.—Total fatty acids and cholesterol were determined together in a portion of the alcohol-ether extract by saponification, extraction of the acidified residue with petroleum ether, and oxidation of an aliquot of the solution with the sulfuric acid-dichromate reagent. Cholesterol was determined colorimetrically in another aliquot, and its oxidation value calculated and subtracted from the oxidation value of the mixture of total lipid, giving the value for total fatty acids. The fact noted above, that the oxidation values for the common fatty acids and also for cholesterol are close together, makes negligible the error of such a calculation.

The procedure was as follows: Portions of the alcohol-ether extract containing about 5 mg. of total lipid (ordinarily 15 to 20 cc. of the extract made as directed above) were measured into 100 cc. Erlenmeyer flasks, 2 cc. of the sodium ethylate added, and the whole evaporated on the water bath to absence of alcohol (odor). The traces of alcohol vapor were swept out of the flask by a gentle current of air, leaving a residue which was pasty but not dry. 1 cc. of dilute sulfuric acid (1 part of concentrated acid to 3 parts of water) was added to render the mixture acid after it was extracted with petroleum ether as follows: The acid mixture was heated on the water bath for 1 minute, and to the hot mixture were added 10 cc. of petroleum ether, which was thereby made to boil. The flask was then rotated gently at the boiling temperature on the water bath for 2 or 3 minutes, after which the solvent was completely poured off from the watery residue into a 25 cc. volumetric flask. The heating and extraction were repeated with portions of about 5 cc. each of petroleum ether, the sides of the flask being washed down and the petroleum ether poured off completely each time, until the volumetric flask was nearly full, after which it was brought to room temperature, filled to the mark, the contents well

mixed, and the flask tightly stoppered. Owing to the marked difference in the surface properties of petroleum ether and water this simple procedure is adequate for complete extraction, since the petroleum ether can be poured off clean from the water each time. All lumps or fragments of suspended matter should disappear under this treatment and the acid water after extraction should be clear and free from particles.

Oxidation.—10 cc. aliquots of the petroleum ether solution containing therefore about 2 mg. of lipid were measured into the 125 cc. glass-stoppered digestion flasks. The solvent was evaporated and the last traces were blown out with a gentle stream of air. To the flasks were then added 5 cc. of the silver reagent (measured carefully because it contains some chromic acid) and then 3 cc. (accurately measured) of the N dichromate, with rotation. A control containing all the reagents except the fatty material was prepared and run along with the samples under exactly the same conditions. Ordinarily, a set consisted of four samples and one control.

After mixing the material well by rotation, the flasks were *loosely* stoppered and set in the oven. After 5 minutes heating they were removed, rotated to stir up the contents, the stoppers *tightly* inserted, and the flasks replaced in the oven for the remainder of the heating period,—a total of 15 to 20 minutes. At the end of this period the flasks were removed from the oven and without cooling 75 cc. of distilled water were added, after which the excess of dichromate was measured by titration as follows:

To the flask were added 10 cc. of 10 per cent potassium iodide and without stirring (which might result in loss of iodine by fuming), the 0.1 N thiosulfate was run in from a burette, the contents of the flask being kept mixed by rotation, at first gentle, then as the iodine content diminished more forceful. The silver causes a white precipitate to form which, however, does not interfere with the color change in the titration or with the endpoint. When the titration was nearly complete a few drops of the 1 per cent starch solution were added and the titration was finished as usual. The titration of the blank was carried out in the same way. The difference between the titrations of the blank and the sample represents the amount of 0.1 N dichromate used by the fatty material in the sample.

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For example, with a sample of oleic acid (theoretical value per mg. = 3.61 cc. of 0.1 N dichromate).

Titration of blank = 33.00 cc. 0.1 N thiosulfate.
" " sample = 23.45 " 0.1 " "
Difference. 9.55 " 0.1 " "
Equivalent to 9.55 cc. 0.1 N dichromate.

Representing $\frac{9.55}{3.61}$ = 2.65 mg. oleic acid.

The digestion mixture should remain definitely brown throughout the heating period, indicating an excess of oxidizing agent. If the mixture becomes green or even shows a marked green tint either when the reagents are first mixed or at the end of the first 5 minutes of heating, it means that the necessary excess of oxidizer is not present. The determination may ordinarily be saved by the addition of a second amount of silver reagent and dichromate and continuing the heating, but another determination should be run as a check with a sufficiently smaller aliquot to make sure that the necessary excess of reagent is present.

Cholesterol.—Cholesterol was determined colorimetrically in a second aliquot of the petroleum ether extract, according to an earlier procedure to which the reader is referred (18). 10 cc. of the extract were measured into a small Erlenmeyer flask and the solvent completely removed by evaporation as before. Chloroform in successive small portions was added, gently warmed to dissolve the residue, and decanted into the 10 cc. glass-stoppered graduated cylinders recommended for this determination. At least three extractions were made, after which the chloroform extract was adjusted to approximately room temperature and made up to the 5 cc. mark. 1 cc. of acetic anhydride and 0.1 cc. of concentrated sulfuric acid were added, the cylinder stoppered, and the substances well mixed. As a standard for comparison 5 cc. of a standard solution of cholesterol containing 0.5 mg. of cholesterol were measured into a similar 10 cc. cylinder, the same amount of acetic anhydride and sulfuric acid added, and the materials well mixed. The cylinders were placed in the laboratory under the same light conditions in which the reading was to be made, left 15 minutes, then the readings made as usual, and the cholesterol value calculated from the reading.

Calculations.

From the two values, total lipid and cholesterol, total fatty acids may be calculated as in the example which follows: 3 cc. of a sample of plasma were extracted with 50 cc. of alcohol-ether mixture. 15 cc. of the extract were taken, saponified, extracted, and the extract made to 25 cc. in petroleum ether. A 10 cc. aliquot of the petroleum ether extract required 7.00 cc. of 0.1 N dichromate for oxidation. Another 10 cc. aliquot was found to contain 0.638 mg. of cholesterol by colorimetric determination.

As noted above 1 mg. of cholesterol requires 3.92 cc. of 0.1 N dichromate for complete oxidation. 0.638 mg. would require then 0.638×3.92 cc., or 2.50 cc. of 0.1 N dichromate. $7.00 - 2.50 = 4.50$ cc. of 0.1 N dichromate for the total fatty acids. The common fatty acids require for complete oxidation amounts of dichromate as follows: palmitic 3.59, oleic 3.61, stearic 3.66. Since the ones present in the largest amounts are oleic and palmitic acids and since oleic acid is generally considerably in excess of all the others, the factor 3.60 is sufficiently close to the truth for practical purposes, especially as it is within 2 per cent of any of the values.

The weight of total fatty acids would then be $\frac{4.50}{3.60} = 1.25$ mg. The final aliquot used corresponds to 0.36 cc. of plasma (3 cc. of plasma dissolved in 50 cc., 15 cc. taken $= \frac{3}{50} \times 15 = 0.9$ cc. of plasma; 0.9 cc. in 25 cc. of petroleum ether, 10 cc. taken $= \frac{10}{25} \times 0.9 = 0.36$ cc. of plasma). The values for 100 cc. of plasma would then be:

Total fatty acids	$\frac{100}{0.36} \times 1.25$	= 347 mg. per cent.
Cholesterol	$\frac{100}{0.36} \times 0.638$	= 177 mg. per cent.
Total lipid,		525 mg. per cent.

A factor for approximate calculations of total lipid may be calculated from the fact that cholesterol as found in plasma is ordinarily close to one-half the value of the total fatty acids. The factor would then be obtained as follows: $2 \times 3.60 + 1 \times 3.92$

$= 3\ x$; from which $x = 3.7$ cc. 0.1 N dichromate; from which and the titration value for total lipid in the above example, the calculated value for total lipid = 524.

Recovery of Added Lipid.

Testing of the method by recovery of added lipid was carried out as follows: Oleic acid as soap solution in alcohol-water mixture was added in measured small amounts to plasma and the plain and the treated plasma were carried through the procedure as directed. Results were obtained as shown in Table III.

TABLE III.
Recovery of Added Fatty Acid.

Lipid content of plasma.	Fatty acid added.	Lipid content of mixture.	Fatty acid recovered.	Fatty acid, per cent recovery.
mg. per cent	*mg. per cent*	*mg. per cent*	*mg.*	
475	200	771	196	98
406	400	807	401	100
417	300	700	283	94
354	184	532	178	97
374	184	550	176	96
381	276	659	278	101
419	368	787	355	96
453	276	712	259	94
493	184	677	184	100
405	184	580	175	95
358	276	644	284	103
405	276	677	272	98

Lecithin.—Determinations of phospholipid (lecithin) may be made in aliquot portions of the alcohol-ether extract according to earlier directions (19). At present the writer has very little to add to what has already been said about this procedure. With regard to the criticism by Baumann (20) that phosphoric acid is lost during the ashing with the Neumann mixture of sulfuric and nitric acid, it may be said that the loss of phosphoric acid is connected largely with the distillation of the sulfuric acid and that if care be taken in the heating to avoid boiling off much of the sulfuric acid, no appreciable loss of phosphoric acid occurs (21). In recent years a number of colorimetric procedures have been offered which have been shown to work well for the deter-

mination of phosphoric acid and which may be applied with suitable precautions to the sulfuric-nitric acid digestion mixture. In the author's experience these methods have no advantage over the nephelometric determinations of phosphoric acid as the strychnine-phosphomolybdate originally described, except that the determinations may be carried out by the use of the colorimeter instead of requiring a special instrument, the nephelometer. The strychnine-phosphomolybdate suspension is especially well adapted for nephelometric work, being finely divided, reasonably stable, and easily reproducible.

Discussion of the Procedure.

Examination of the literature and personal experience bring the conviction that accurate determination of the fatty substances of the tissues and fluids is never an easy procedure, and particularly is this true of the micro methods. The directions given above are simple and when properly carried out are adequate, but exacting quantitative care must be taken at every step. Practice determinations on known solutions until accuracy is attained should precede all serious work. The following notes on the procedure are intended to be helpful.

Preparation of Sample.—The amount of plasma and alcohol-ether mixture recommended—3 cc. of plasma in 50 cc. of alcohol-ether mixture—will yield enough filtrate for duplicate determinations of total lipid and cholesterol (therefore also of total fatty acid) or for single determinations of these substances and lecithin. If duplicate determinations of all these are required (which is advisable), more plasma and alcohol-ether mixture should be used—5 cc. of plasma in 100 cc. of alcohol-ether mixture. On the other hand very good duplicate measurements of total lipid, cholesterol, and total fatty acids may be obtained from 1 cc. of plasma in 50 cc. of alcohol-ether mixture or single measurements from 0.5 cc. of plasma, since the oxidative procedure as outlined gives good results with 1 mg. of lipid. It is recommended, however, that the procedure be followed as directed since thereby a sufficient titration difference is obtained to minimize the effect of errors inherent in titration. The completeness of the extraction by this short procedure depends upon the fact that the protein is precipitated in finely divided flocculent form, so that all parts of

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the precipitate are in close contact with the large excess of solvent, with the result that solution of the soluble material takes place at once. If large clumps of precipitated protein are allowed to form by improper addition of plasma to the solvent, complete extraction is difficult or impossible. In preparing the alcohol-ether sample the flask should be as nearly full as is safe to avoid boiling over during the heating, since it is this solvent and not what is added later that is responsible for the extraction. The flasks should be at least three-fourths full when the plasma is run in. The alcohol-ether mixture readily superheats, and, if care is not taken during the heating to keep the material in the flask in considerable motion, some may be lost by explosive boiling.

Saponification and Second Extraction.—In the determinations as carried out, *i.e.* determination of total fatty acid and cholesterol together, saponification is not strictly necessary. Values in fair agreement and about 10 per cent higher (because of the glycerol and choline in the lecithin) are obtained by merely evaporating the alcohol-ether mixture and extracting the residue with warm petroleum ether. Mechanical difficulties—the presence of flecks of insoluble material, hard to separate from the petroleum ether, render this procedure difficult so that saponification is recommended.

At the end of the saponification the traces of alcohol are completely removed (by air current) since otherwise the alcohol passes into the petroleum ether and may escape removal during the evaporation of that solvent and so increase the values obtained by the oxidation. In driving off the alcohol the residue is brought almost to dryness and thereby there may be formed lumps of material which contain lipid and which must be completely broken up during the acidification and extraction before all the lipid can be dissolved. In the later treatments with petroleum ether care should be taken that the solvent reaches all parts of the flask where fatty material is likely to be. If all has gone well the treatment with successive portions of warm petroleum ether should result in rapid and complete extraction, the whole extraction not occupying more than 20 minutes. The acid water after extraction should be clear and free from suspended material. It may be shown to contain reducing substances (sugar ?) and other alcohol-soluble organic and inorganic compounds; hence the use of petrol-

eum ether as a solvent since it is a more specific solvent for lipid than ether or chloroform and also does not dissolve appreciable amounts of water which in turn would carry with it water-soluble substances. The fact that petroleum ether is not as good a lipid solvent as ether or chloroform is allowed for in the excess used. However a fact should be noted with regard to petroleum ether which is often forgotten by workers in the lipid field and that is that certain of the fatty acids, as for example the hydroxy acids, are almost insoluble in it. Whether these acids are present in blood plasma is unknown, but it can easily be demonstrated that a fatty acid mixture from blood, which may be entirely soluble when freshly prepared, will become partly insoluble in the same solvent when left exposed to air or even when allowed to stand in petroleum ether.

Oxidation.—Since the determination depends on the amount of oxidizing agent destroyed, care must be taken at all stages to exclude oxidizable foreign substances and also to allow for the possible effect of the oxygen of the air whether as such or in solution in the solvents used. Fortunately direct oxidation by the oxygen of the air appears to be largely negligible.

The petroleum ether is allowed to evaporate on the steam bath and the fumes of solvent completely removed from the flask by a gentle current of air, since, if not completely removed, they are oxidized by the reagent and so result in high values. Because of the possibilities of oxidation the air current should be used sparingly but all the solvent must be removed. Odor is here not a safe guide since the purified solvent has little odor. As a guide in the removal the following has been found useful. When observed against a light, the fumes of petroleum ether can be seen pouring out over the rim of the flask when the air current is applied and the air current should be kept up for about a half minute after the fumes have disappeared. The possibility of oxidation of the lipid by this procedure was tested by the use of a current of nitrogen instead of air but no consistent difference could be noted.

Various modifications of the oxidizing mixture were tried, from undiluted sulfuric acid-dichromate reagent with and without silver, to dilution with water up to 5 times. The undiluted reagent decomposes readily with heat, especially the reagent con-

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taining silver, and it has not been found possible to control the decomposition. Dilution with water stabilizes the reagent, but too much water reduces its efficiency until complete oxidation is difficult or impossible. 3 cc. of water to 5 cc. of sulfuric acid were found to be the most useful dilution, and this was brought about in the method by the addition of 3 cc. of N dichromate to the sulfuric acid-silver reagent. When the oxidizing solution is added to the lipid mixture as in the method described above, most of the oxidation takes place rapidly so that by the time the solution has cooled about 60 per cent of the oxidation has been completed. Leaving the solution for 24 hours at room temperature brings the percentage up to about 80. Heat, therefore, is necessary to complete the reaction in reasonable time. Various combinations of time and temperature were tried and several were found adequate, but the two combinations recommended, *i.e.* 124° for 15 minutes and 90° for 1 hour, seemed best adapted to ordinary laboratory conditions and to the peculiarities of the reagent.

The temperature-time factor is critical, especially at the higher temperature, and must be checked closely. The reagent in order to act effectively must be in a state of slight decomposition, and while the actual decomposition is insignificant during the time of heating, it is nevertheless present and apparently does not proceed at the same rate in control and test solutions, which may be because the amount of undecomposed dichromate is not the same in the two solutions as the reaction proceeds. This may also be the reason why a large excess of dichromate is required—3 or 4 times the amount actually reduced. As noted above there is normally a slight decomposition of the reagent during the heating, as may be shown by differences in titrations of the heated and unheated blanks. Ordinarily the difference should not exceed 0.3 or 0.4 cc. of 0.1 N thiosulfate, and if it exceeds that value the temperature is probably too high. At water bath temperature the decomposition of the reagent in the time required is less but still noticeable.

Reoxidation by the oxygen of the air is prevented by the use of glass-stoppered flasks and by the procedure as follows: Since the greater part of the chromic acid oxidation takes place quickly, the flasks are loosely stoppered during the first period of heating to allow the escape of gases and are then tightly stoppered.

When the heating period is complete, the digestion mixture is at once diluted with water without cooling so as to prevent oxygen absorption by the concentrated reagent. The possibility of oxidation of the reduced reagent by the oxygen in the distilled water (22) has been tested by the use of water which has been freshly boiled and cooled without stirring. No consistent difference could be observed. That slow reoxidation of the diluted solution does not take place to any appreciable extent, is shown by the fact that the solutions after dilution may stand 2 or 3 hours without changes in their titration values. Standing overnight will, however, result in appreciably lower values.

Titration.—The addition of 10 cc. of potassium iodide results in the liberation of so much iodine that there is danger of loss through fuming, to avoid which the iodide is not added until one is just ready to titrate. It is added with only enough rotation to mix the solutions, and much stirring or shaking is avoided until the concentration of the iodine is considerably reduced by titration. It is believed that the chances of error by this procedure are less than by the alternative one of large dilution involving transference to another vessel. The muddy greenish brown solution becomes during titration lighter and lighter in color until at completion it is a light blue. The time to add the starch is just before the disappearance of the last tint of yellow. After the addition of starch the end-point is perfectly plain even in the presence of the white precipitate of silver salt.

Corrections.—As may be seen from Table I, absolute values for oxidation are never quite attained, the shortage being about 3 per cent. In the whole procedure on blood plasma, consisting of extractions and oxidation, the shortage is about 5 per cent (see Table III). Correction may be made on this basis, but, inasmuch as there is still considerable uncertainty as to the nature of blood lipids, the value of the correction may be questioned except in cases such as in the testing of the procedure by recovery of added lipid, the nature of which is known.

Cholesterol.—A yellowish tint to the green color in some of the samples seems unavoidable. In most cases it is not sufficient to interfere with the readings but occasionally it is, and in these instances it has been found advantageous to use the red light-filter that is supplied with the colorimeter. The readings are

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rendered much easier thereby and no appreciable difference in values has been found.

Comparison of results for fatty acids and cholesterol by the above procedure with those obtained nephelometrically has thus far not shown any essential difference between the two methods, although the results by the oxidative method were slightly higher. Discussion of this point together with others connected with the determination of small amounts of lipids in blood is reserved for a later communication.

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THE LIPID DISTRIBUTION IN NORMAL AND ABNORMAL LIVER TISSUES.

II. THE EFFECT OF INSULIN ON THE LIPIDS OF RABBIT LIVER.

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In a recent paper (1) we have discussed certain data regarding the lipid distribution in normal and abnormal liver tissues. In this paper a theory was suggested; namely, that in normal liver tissue there exists a certain definite lipid distribution or equilibrium

$$\text{phospholipid} \rightleftharpoons \text{neutral fat}$$

Normal liver tissue from rabbits, dogs, beef, and humans has shown this relation to be approximately

$$\underset{55 \text{ to } 65 \text{ per cent}}{\text{phospholipid}} \rightleftharpoons \underset{35 \text{ to } 45 \text{ per cent}}{\text{neutral fat}}$$

Examination of abnormal liver tissue, such as that occurring in fatty degeneration and fatty infiltration has shown that this equilibrium state is materially altered, the equilibrium relation being markedly shifted to the right.

Paper I of this series (1) showed the above mentioned relation clearly but we were interested to ascertain whether abnormal conditions regularly caused a decrease in phospholipid content. It is commonly accepted that an interrelationship exists between the combustion of carbohydrate and of fat within the organism, and this idea was the basis of this paper. Injection of insulin into the blood stream rapidly lowers the blood sugar. What effect has insulin upon the lipids of the various tissues—for instance the liver? Omura and Nitta (2) have shown that after insulin injection the fatty content of heart, kidney, and skeletel muscle increases, while that of the liver tissue decreases. Liu and Mills (3) have investigated the effect of insulin on blood cholesterol, fat, and sugar in nephrosis and find that insulin given three times daily before meals causes a decrease of both fat and cholesterol in the blood. Lombroso (4) and Sunzeri (5) find also that insulin injection decreases the fatty content of the liver. Macleod (6) in his monograph says with regard to the effect of insulin on the fat of the liver

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and blood, that when insulin is given, the fat invasion of the liver becomes much less, and gives tables in support of the contention.

These findings are in direct accordance with our data, but the decrease of fatty content of the liver is not marked enough in every case to draw any decided conclusion. In many cases there is a decided decrease in the fatty content after insulin injection over a period of time, but in other cases, the decrease is hardly evident. Believing that insulin injection must affect the liver lipids in some manner, we have determined the lipid distribution and characteristics of the lipids of rabbit livers.

EXPERIMENTAL.

The experimental work was of two kinds; first, the establishing of control conditions and second, the treatment of the animals

TABLE I.

Lipid Material of Normal Rabbit Livers.

Series No.*	1	2	3	4	5	6
Total lipids, *per cent*	2.53	2.36	2.99	2.17	2.64	3.00
Phospholipids, " "	56.20	65.70	53.25	60.15	59.00	55.50
Neutral fat, " "	43.80	34.30	46.75	39.85	41.00	44.50

* The controls were made in series, four to five animals being used in each. The results for each series were then averaged; they are given in the table.

(rabbits in this case) with insulin under varying conditions. For controls some twenty animals were used. The animals were allowed to adjust themselves to our conditions of housing and diet. All the animals used for *controls* and for *treatment* with insulin were allowed no food for 24 hours previous to treatment. For the work outlined in this paper, the same conditions were maintained as for the method of insulin assay of the Toronto workers. After 24 hours fasting, the control animals were killed with chloroform, the livers removed, and examined chemically for lipid materials. Table I gives the results obtained for normal rabbit liver lipids.

Normal animals fasted for 24 hours previously were treated with insulin under the following conditions: (1) series of animals

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injected with 3 units of insulin per day for 21 days; ordinary diet and no loss of appetite observed; (2) series of animals injected with 3 units of insulin; killed after 6 hours (after peak action had passed); no convulsions; (3) series of animals injected with 3 units of insulin; killed during convulsions (peak of reaction); (4) series of animals injected with 8 units of insulin each day for 9 days. These animals however were allowed as much food as they cared for. They were killed on the 9th day. No convulsions occurred; no loss of appetite was observed; (5) series of animals given orally phosphorus in olive oil; killed after 36 hours; liver and heart showed distinct signs of fatty degeneration changes.

In each series the liver tissues were removed, extracted with alcohol in a manner previously described (7), and the lipid material examined quantitatively. The results for each series were then averaged and are given in this form in Table II.

TABLE II.

Lipid Material of Livers from Insulin-Treated Rabbits.

Series No.	1	2	3	4	5
Total lipids, *per cent*	1.16	3.63	3.37	2.84	2.64
Phospholipids, " "	16.50	38.20	29.50	43.00	3.58
Neutral fat, " "	83.50	61.80	70.50	57.00	96.42

From Tables I and II we can state the following: (*a*) that the equilibrium equation for normal rabbit liver tissue is

$$\underset{\text{55 to 65 per cent}}{\text{phospholipid}} \rightleftharpoons \underset{\text{35 to 45 per cent}}{\text{neutral fat}}$$

(*b*) that the equilibrium equation for liver tissues of insulin-treated rabbits is

$$\underset{\text{16 per cent to normal}}{\text{phospholipid}} \rightleftharpoons \underset{\text{84 per cent down to normal}}{\text{neutral fat}}$$

(*c*) that with identical unitage of insulin per kilo of body weight, the phospholipid-neutral fat relation is shifted to the right more markedly when the animal is killed at the peak of insulin reaction than when killed several hours after peak reaction. This fact

is readily seen in Series 2 and 3 of Table II. In Series 3, killing the animals at the peak reaction gave a phospholipid-neutral fat ratio of 29.5 : 70.5. In Series 2, or considerably past the peak reaction, the ratio was 38.2 : 61.8; (*d*) that under prolonged insulin treatment as illustrated in Series 1, the total lipid content is sharply decreased; (*e*) that as pointed out previously (7) fatty degeneration caused by phosphorus poisoning (Series 5) alters the phospholipid-neutral fat ratio from the normal state to one of 3.58 : 96.42.

TABLE III.

Iodine Values of Fatty Acids of Normal Rabbit Liver Lipids.

	per cent
Phospholipid fraction.	
Iodine value of fraction	68.7
" " " mixed fatty acids	102.5
" " " liquid acids	200.0
4-Bond acids in " "	12.5
Solid fatty material.	
Iodine value of fraction	69.2
" " " mixed fatty acids	84.5
" " " liquid acids	180.5
4-Bond acids in " "	Trace.
Liquid fatty material.	
Iodine value of fraction	83.8
" " " mixed fatty acids	72.0
" " " liquid acids	218.0
4-Bond acids in " "	2.6

We have been able to find few conclusive references with regard to the fate of liver lipids during insulin treatment. Insulin investigation has been confined mainly to carbohydrate metabolism and little attention paid to the action of this drug upon body lipids. Macleod ((6) p. 81) points out that in depancreatized animals enlarged livers may result and the liver tissue may show an extreme degree of fatty deposition and cellular degeneration.

Unsaturation of Fatty Acids in Lipids.

It was of interest to study quantitatively the degree of unsaturation of the fatty acids that make up the lipids of normal rabbit liver tissue. This was done in order that a general comparison

might be made with the fatty acids of beef liver lipids. In order to obtain accurate data, it was necessary to have some twenty rabbit livers. These livers in general weigh from 30 to 60 gm. each and usually contain 2 to 3 per cent of lipid material. After death the livers were quickly removed, passed through a meat chopper and then dehydrated with alcohol. The lipid material was extracted according to the well known method of Bloor (8), the various lipids separated, saponified, and the fatty acids obtained. Iodine values were made on the fraction, the mixed fatty acids, and on the liquid fatty acids. The percentage of 4-bond acids was determined by brominating the ether solution of the liquid acids. The experimental data are given in Table III.

Table III shows that the fatty acids of the extractable lipids of normal rabbit liver tissue are of about the same degree of unsaturation as those of beef liver lipids (1). It was pointed out in the case of beef liver lipids that the 4-bond acids were some 34.5 per cent of the liquid fatty acids of the phospholipid fraction. In the rabbit liver lipids the 4-bond acids were 12.5 per cent of the liquid acids in the phospholipid fraction, a trace in the solid fatty material fraction, and 2.6 per cent in the liquid fatty material fraction. In all other respects, the fatty acids seem to parallel those of beef liver lipids, the iodine values being only slightly lower.

CONCLUSION.

The effect of insulin upon the lipid distribution of rabbit liver tissue has been investigated and the following changes were observed. Over a long period of time, insulin causes a decrease in the total lipid content of the liver. Over a very short period, the insulin causes a decided change in the phospholipid-neutral fat relation. If the animal is killed at the peak of the insulin action, we find less phospholipid in the liver tissue than if the animal is killed several hours past the peak reaction.

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STUDIES ON GOSSYPOL.

III. THE OXIDATION OF GOSSYPOL.

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(Received for publication, January 25, 1928.)

Attempts to obtain information concerning the nature of the structure of gossypol have been limited to the experiments of Carruth (1). This investigator studied the effects of alkali fusion and several oxidizing agents upon gossypol but did not succeed in obtaining any pure crystalline derivatives or other evidence of a clarifying character.

One of the methods used in this laboratory for studying the nature of the gossypol molecule has been to investigate the effects of oxidizing reagents upon the substance. The results as a whole have been more or less discouraging, for with most reagents and methods tried either nothing at all or only amorphous or tarry products having no tendency to crystallize were obtained. With potassium permanganate, however, conditions were found which yielded certain products that have a significant bearing upon at least one phase of the problem. In these experiments gossypol dissolved in slightly more than 2 mols of sodium hydroxide was treated at 0° with potassium permanganate in the proportions of 1 part of gossypol to 5 parts of permanganate. The reaction was quite energetic and terminated in a short time. The manganese dioxide formed was removed by filtration, and the filtrate was made acid with sulfuric acid. In addition to the evolution of carbon dioxide there was evidence of the presence of volatile fatty acids in the reaction mixture. The solution was therefore steam-distilled, and the distillate was examined by the method of Dyer

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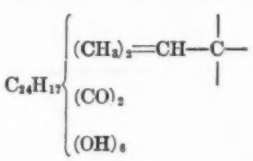
(2) for fatty acids. From an examination of the distillation curve obtained it was possible to make certain deductions concerning the nature of the acid distillate. First, as the curve was not a straight line, more than one acid was present. Second, in the beginning the curve passed between the distillation curves of *n*-butyric and isobutyric acids which thus indicated the presence in the mixture of the latter. Third, as the curve crossed the distillation curve of acetic acid, the presence of formic acid was shown, and finally, the slope of the curve strongly indicated the presence of acetic acid. These conclusions were verified by applying specific confirmatory tests and by preparing certain characteristic derivatives of the respective acids. It was further demonstrated that the acids mentioned were the only ones present. A quantitative estimation of the different acids was also made.

The existence of formic acid under the conditions of the experiment may appear at first to be paradoxical; but when it is considered that the potassium permanganate was not used in excess, it is evident that some of this acid could easily escape oxidation. It is believed, on the basis of certain experiments which will be reported in a subsequent communication, that the formic acid was derived from the carbonyl groups which have previously been shown to be present in gossypol (3). It would be unwise at this time to attribute the formation of acetic acid to any definite structure in the original material, but the identification of isobutyric acid as a decomposition product seems to indicate conclusively that there is a side chain in the gossypol molecule involving at least the group $(CH_3)_2{=}CH-\overset{|}{\underset{|}{C}}-$. It is quite possible that this group is joined to the remainder of the molecule through a double bond and that the potassium permanganate functions here as it commonly does with double bonds resulting in a rupture of the chain with the formation of acids. This would indicate a certain amount of unsaturation in the compound. Evidence is at hand to support this but at present the information available as to the extent of this property is not satisfactory.

Besides the acids enumerated above, another product was secured by extracting with ether the residue obtained after removing the volatile fatty acids from the reaction mixture. This consisted

of a syrup showing little tendency to crystallize, although a few small crystals were formed. However, as the quantity of the product was quite small, there seemed to be little hope of accomplishing much with it without the sacrifice of large quantities of starting material. For the present, therefore, its investigation will not be undertaken.

With the information now at hand the knowledge of the structure of gossypol may be indicated as follows:



EXPERIMENTAL.

5 gm. of gossypol were dissolved in 200 cc. of water containing slightly more than 2 mols of sodium hydroxide. This was slowly treated with 25 gm. of potassium permanganate dissolved in 400 cc. of water, both solutions having previously been cooled to 0°. The reaction was quite vigorous and apparently terminated in a short time. After the mixture was allowed to stand overnight, the manganese dioxide was filtered from the liquid and thoroughly washed with water. The filtrate and washings were then made acid to Congo red with sulfuric acid, which caused the evolution of carbon dioxide. The volatile fatty acids were obtained from this acidified solution by steam distillation. The distillate collected required for neutralization 194 cc. of 0.1 N sodium hydroxide. The resulting solution was evaporated to approximately 100 cc. upon a steam bath, transferred to a distilling flask, acidified with slightly more than the calculated quantity of sulfuric acid to free the organic acids, and again steam-distilled. The first 400 cc. of distillate were collected separately and neutralized with sodium hydroxide. There were required for this purpose 107 cc. of 0.1 N alkali. The resulting solution of sodium salts was evaporated to dryness. The residue was transferred to a distilling flask with sufficient water to make a volume of 150 cc., acidified, and distilled according to the method of Dyer (2) for the identification of volatile fatty acids. The results are recorded in Chart I.

As mentioned before, a study of the distillation curve thus obtained indicated that formic, acetic, and isobutyric acids were in the mixture. In order to verify these conclusions more material

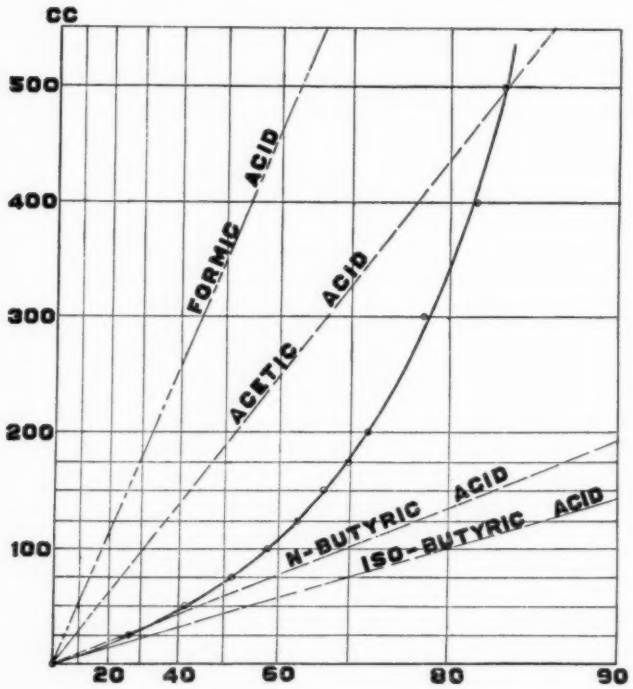


CHART I. Distillation curve (according to the method of Dyer) of the volatile fatty acids obtained by alkaline permanganate oxidation of gossypol. The distillation curves of formic, acetic, *n*-butyric, and isobutyric acids are given for comparison. The abscissas represent the percentages of the total acids distilled (107 cc. of 0.1 N acid) which were found in definite fractions of the distillate, while the volumes in cc. of these fractions are represented by the ordinates.

was prepared by working up 10 gm. of gossypol as outlined before. The sodium salts which resulted from neutralizing the distillate with sodium hydroxide were obtained by evaporating their solu-

tion to dryness on a steam bath. This material was dissolved in a small quantity of water, made acid to Congo red with sulfuric acid, and steam-distilled. The distillate was collected in 3 portions for the purpose of making a rough separation of the different acids. The first fraction contained rather large proportions of the isobutyric acid. The second contained relatively large quantities of acetic acid, while the last fraction contained much formic acid. These fractions were separately neutralized with sodium hydroxide and evaporated to dryness. The sodium salts thus obtained were subjected to individual tests for the respective acids.

Upon treating the sodium salts of the first fraction with sulfuric acid, the odor of isobutyric acid became very strong. The ethyl esters obtained by treating the sodium salts with alcohol and concentrated sulfuric acid also had the characteristic odor of ethyl isobutyrate. The *p*-bromophenacyl esters of the acids were then prepared according to the directions of Judefind and Reid (4). The crude product was subjected to fractional crystallization, and in this way several fractions melting between 74–76° were obtained. These were united and recrystallized giving a substance which melted at 76° (uncorrected). When this was mixed with a pure sample of the isobutyl ester which melted at 76.5–76.8° no depression of the melting point occurred; *i.e.*, the melting point of the mixture was 76.5°. The optical properties of the preparation were identically the same as those of a pure sample of isobutyl *p*-bromophenacyl ester. These data were determined by Mr. George L. Keenan of the Food, Drug and Insecticide Administration of the Department of Agriculture who reported as follows: "The material consisted of thin, colorless, plate-like jagged rods some of which were six-sided. The most distinctive index of refraction was 1.565 frequently shown lengthwise on the rods. The other indices were not determined as the material was slightly soluble in oily immersion liquids."

For the identification of acetic acid, the sodium salts obtained from the second fraction were converted into the *p*-toluides by the method given by Mulliken (5). The material obtained was purified by recrystallization from benzene. It melted at 148.5° (corrected), and no depression of the melting point occurred when the substance was mixed with a pure sample of aceto-*p*-toluide.

Formic acid in the last fraction was identified by its capacity for

reducing potassium permanganate in the cold, mercuric chloride to mercurous chloride, silver nitrate to silver when warmed or upon standing, and also by applying the specific tests recommended by Mulliken ((5) p.83) for formic acid. These tests consisted of reducing mercuric oxide to mercury and decomposing formic acid with sulfuric acid with the evolution of carbon monoxide which was identified by its property of burning with a blue flame.

To demonstrate the absence of other acids which might have been in the mixture, the neutralized distillate which resulted from the running of the distillation was fractionally precipitated with silver nitrate. The first crop of silver salts obtained contained 64.48 per cent of silver, showing that it was practically pure silver acetate (theory 64.64 per cent). If propionic, *n*-butyric, or other higher members of the fatty acid series had been present, these would have precipitated first, giving a fraction of silver salts with a lower silver content.

Estimation of Fatty Acids.

Another experiment was undertaken for the purpose of estimating the quantity of each of the fatty acids in the reaction mixture. For this purpose 5 gm. of gossypol were treated as has been described, except that the distillation of the acidified mixture was continued until all the volatile fatty acids passed into the distillate. For neutralization of these acids 232 cc. of 0.1 N alkali were required. The resulting solution of sodium salts was evaporated almost to dryness, diluted to 300 cc., and one-half of the liquid was transferred to a flask with a reflux condenser. Sulfuric acid was added to this in sufficient quantity to liberate the organic acids and then the acidified solution was boiled with an excess of precipitated mercuric oxide until effervescence ceased, which indicated the complete destruction of formic acid. The resulting mixture was then cooled and transferred to a distilling flask. Sufficient sulfuric acid was added to dissolve all the unchanged mercuric oxide and to render the solution strongly acid. The acetic and isobutyric acids remaining in the mixture were then completely recovered by steam distillation, and titrated. The distillate required for neutralization 85.6 cc. of 0.1 N alkali; but as only one-half of the original acid mixture was thus treated, 171.2 cc. of 0.1 N acid represented acetic and isobutyric acids obtained from 5 gm.

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of gossypol. The difference between 232 and 171.2, or 60.8 cc. of 0.1 N acid, represented formic acid. This is equivalent to 0.280 gm. of the latter.

The neutralized distillate containing the acetic and isobutyric acids was evaporated, acidified, and distilled according to Dyer (2) in order to obtain the distillation constant of the mixture. This was found to be 56.3, and from this value the quantity of each component in the solution was calculated. The results were acetic acid, 0.247 gm.; isobutyric acid, 0.391 gm. Thus the total acids formed by the oxidation of 5 gm. of gossypol were formic acid, 0.280 gm.; acetic acid, 0.494 gm.; and isobutyric acid, 0.782 gm. The yield of isobutyric acid corresponded to 92.1 per cent of that required by theory, if it is assumed that each mol of gossypol produced 1 mol of isobutyric acid.

SUMMARY.

1. Upon oxidation of gossypol with alkaline permanganate, formic, acetic, and isobutyric acids have been identified as reaction products.

2. The quantities of the respective acids formed by the reaction were determined and in the case of isobutyric acid there was obtained 92.1 per cent of the amount required by theory if it is assumed that 1 mol of gossypol yielded 1 mol of acid.

3. Under the conditions of the experiment the presence of isobutyric acid as a decomposition product of gossypol indicates the presence in the gossypol molecule of a side chain consisting of at least the isobutyl group.

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STUDIES IN THE SYNTHESIS OF HIPPURIC ACID IN THE ANIMAL ORGANISM.

VII. A COMPARISON OF THE RATE OF ELIMINATION OF HIPPURIC ACID AFTER THE INGESTION OF SODIUM BENZOATE, BENZYL ALCOHOL, AND BENZYL ESTERS OF SUCCINIC ACID.

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(Received for publication, January 30, 1928.)

The discovery of the antispasmodic action of benzyl alcohol and its esters has stimulated the study of the metabolism of substances of this type. Since the antispasmodic effect of the benzyl esters is due, according to some observers at least, to the action of the benzyl alcohol liberated by their hydrolysis, their relative efficiency as antispasmodics should be related directly to their relative rates of hydrolysis in the animal organism. Since benzyl alcohol has been known to be oxidized rapidly to benzoic acid in the animal organism, and the latter, after conjugation, to be excreted, largely as hippuric acid, it seemed probable that the relative rates of hydrolysis of various benzyl derivatives of therapeutic interest could be determined by a comparative study of the rates of formation and elimination of hippuric acid after their administration.

The following experiments have therefore been undertaken to study the rates of hydrolysis of two typical benzyl esters, dibenzyl succinate and sodium benzyl succinate, by determinations of the rates of excretion of hippuric acid following their ingestion by rabbits. As control experiments, benzyl alcohol and sodium benzoate have also been administered. Each of these compounds represents a distinct stage in the metabolic path of the benzyl esters prior to their elimination as hippuric acid. Thus the benzoate after absorption requires only mo-

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bilization (or synthesis) of glycine and conjugation before the elimination of hippuric acid. Benzyl alcohol must be oxidized to benzoic acid before hippuric acid synthesis, while hydrolysis of the esters must first occur, after which the oxidation of the benzyl alcohol and the conjugation of the resultant benzoic acid can be effected.

In selecting for study the benzyl esters of succinic acid, we have been influenced not only by their therapeutic use, but also by the studies of the enzymatic hydrolysis of the esters of succinic and malonic acids *in vitro*. It has been shown (1) that the diethyl esters of these acids were readily hydrolyzed *in vitro* by the lipase of hog liver until an equilibrium was reached, which corresponded to the removal of one ethyl group. The monoethyl ester of malonic acid and the potassium salt of this ester were not acted upon by liver lipase. Kastle (2) has also observed that the metallic salts of the monoethyl esters of the dicarboxylic acids, *e.g.* sodium ethyl succinate, were not split by the lipase of the liver. That this was not the case for the salts of the monoethyl esters of all the dicarboxylic acids was demonstrated by the easy cleavage of monoethyl adipate and sodium ethyl adipate by liver lipase (3). Howard (4), in his studies of the lipolytic activity of pancreatic extracts on dibenzyl succinate, concluded that this ester also was split *in vitro* only to the monobenzyl ester. In view of these *in vitro* studies of the hydrolysis of esters of succinic acid by pancreatic and liver lipases, it has seemed of special interest to study the hydrolysis *in vivo* of the di- and monobenzyl esters of succinic acid as evidenced by the elimination of hippuric acid after their enteral administration.

EXPERIMENTAL.

Male rabbits of medium weight (2.0 to 3.0 kilos) were maintained on a constant diet of 75 gm. of oats and 100 gm. of cabbage throughout the entire experimental period. The urine was collected in 24 hour periods or on the days when the substances, whose behavior was under study, were ingested, in two periods of 6 and 18 hours each respectively. Urine was collected from the cage and from the bladder by gentle pressure on the abdominal wall. The substances fed were administered in solution through a stomach tube. Since dibenzyl succinate is not water-soluble,

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it was fed as a suspension in water. In some of the later experiments, a better suspension was obtained by the use of gum acacia and a small amount of sodium taurocholate. The utilization of the ester was the same in each case. The amount of the benzyl derivatives fed was equivalent to approximately 450 mg. of benzoic acid per kilo of body weight, an amount of benzoic acid which can be administered to rabbits repeatedly without causing loss of appetite, diarrhea, or other toxic effects. A period of at least 3 days elapsed between successive feedings of the compounds studied.

It is evident that the main interest in these studies lay in the determination of the elimination of the benzoic acid formed by oxidation of the benzyl group. It would accordingly have seemed logical to use a method of analysis which should determine *total* benzoic acid, as, for example, the well known method of Folin and Flanders (5), in which, after hydrolysis with alkali, other organic material is oxidized by nitric acid and copper nitrate and the benzoic acid is extracted with chloroform and titrated. Control experiments (unpublished) by Horst, Scott, and one of us (L.) showed that in this method as applied to fluids containing benzyl alcohol or esters, a small but significant amount of benzoic acid was formed by the oxidizing action of the reagents used. We have therefore determined *hippuric acid* by the extraction method of Griffith (6). Since, in this method, hippuric acid is calculated from the nitrogen content of the ether extract, after removal of traces of urea, it is evident that benzyl esters or alcohol, which contain no nitrogen, cannot interfere, even if present and extracted by the ether. The objection might be raised that other forms of conjugated benzoic acid or free benzoic acid might also be present in the urine and that our results therefore would not include all the benzoic acid formed and eliminated. However, as has been shown by Griffith (6) and confirmed in this laboratory (unpublished), if rabbits are fed glycine with moderate doses of sodium benzoate, the amount of benzoic acid eliminated in the urine other than that conjugated as hippuric acid is very slight. This ready synthesis of hippuric acid has led us to choose rabbits as our experimental animals and to feed with the benzyl derivatives or sodium benzoate an amount of glycine equivalent to 3 times the amount of benzoic acid theoretically obtainable from the sub-

stance fed. We believe, therefore, that under the experimental conditions stated (moderate doses of benzoic acid or its equivalent and ingestion of 3 equivalents of glycine) the hippuric acid elimination is a fair measure of the benzoic acid formed and eliminated.

TABLE I.

	Compound fed.	Amount fed expressed as equivalent dose of benzoic acid per kilo of body weight.	Experiment No.	Extra benzoic acid excreted (as hippuric acid) expressed as percentage of ingested benzoic acid.		
				6 hr. period.	18 hr. period.	24 hr. period.
		mg.				
Rabbit D; weight 2.00 kilos.	Benzyl alcohol.	448	D 11	65.0	3.1	68.1
		450	D 15	72.0	5.3	77.3
	Sodium benzyl succinate.	450	D 23	43.6	2.2	45.8
		450	D 29	38.7	1.2	39.9
		450	D 33	34.4	5.7	40.1
	Dibenzyl succinate.	450	D 45	16.7	37.2	53.9
		460	D 54	18.6	22.9	41.5
	Sodium benzoate.	471	D 19	75.1	1.7	76.8
		450	D 37	56.9	4.4	61.3
		450	D 58	59.2	1.8	61.0
Rabbit E; weight 2.25 kilos.	Benzyl alcohol.	450	E 11	51.8	0.0	51.8
		450	E 15	65.0	6.3	71.3
Rabbit F; weight 2.8 kilos.	Sodium benzyl succinate.	450	F 25	39.0	11.4	50.4
		410	F 29	38.3	5.0	43.3
		410	F 33	46.3	0.0	46.3
	Dibenzyl succinate.	450	F 41	12.9	39.3	52.2
		450	F 45	35.1	36.0	71.1
		450	F 54	28.2	43.4	71.6
	Sodium benzoate.	450	F 37	69.0	15.3	84.3
		466	F 58	70.0	15.1	85.1

DISCUSSION.

The results of typical experiments with three rabbits are presented in Table I together with a summary of the average results of all the experiments in Table II. The "extra" benzoic acid

eliminated (as hippuric acid) in each period is calculated in terms of the theoretical amount derived from the weight of the substance administered. In calculating the extra benzoic acid elimination, the average amount of benzoic acid (as hippuric acid) excreted on the normal days has been deducted from the values obtained on the experimental days. This normal elimination has been relatively constant for each animal and has usually amounted to somewhat less than 100 mg. in 24 hours under the dietary conditions of our series.

In harmony with previous observations (6, 7), after the ingestion of sodium benzoate, the maximal excretion of hippuric acid

TABLE II.

Summary of All Experiments.

Average amount of extra benzoic acid excreted (as hippuric acid) expressed as percentage of the amount of benzoic acid theoretically obtainable from the substances administered.

Compound fed.	Total No. of experiments.	Elimination.		
		6 hr. period.	18 hr. period.	24 hr. period.
		per cent	*per cent*	*per cent*
Sodium benzoate	13	69.5	9.5	79.0
" benzyl succinate	12	38.8	6.3	45.1
Dibenzyl succinate	8	24.3	36.6	60.9
Benzyl alcohol	6	65.7	7.1	72.8

was found to occur within 6 hours after feeding, an average of slightly over 69 per cent of the amount fed being present in the urine secreted during that period. After the ingestion of benzyl alcohol, the excretion of hippuric acid was likewise very rapid, the average amount excreted in the first 6 hours being only slightly less than that excreted after benzoate ingestion (65.7 per cent). This is in accord with the observations of Snapper and coworkers (8), who concluded that in man the oxidation of the benzyl alcohol occurred so rapidly that the rate of elimination of hippuric acid was essentially the same, whether sodium benzoate or benzyl alcohol was fed. Our results would lead us to a similar conclusion; namely, that in the rabbit the velocity of oxidation of benzyl

alcohol is *at least as great as* the velocity of hippuric acid synthesis and excretion.

The behavior of the two esters was markedly different. Since the dibenzyl ester is not water-soluble, it might have been anticipated that hydrolysis in the intestine would be slower and that greater loss through the feces would occur, than in the case of the sodium benzyl succinate which is soluble. It was noted that although the excretion of hippuric acid in the urine *in the 6 hours immediately following feeding* was always greater with the salt of the monobenzyl ester than with the dibenzyl ester, the *total recovery as hippuric acid in the 24 hour period* was always less. The elimination as hippuric acid after oral administration of dibenzyl succinate, although delayed so that maximal excretion occurred later than during the first 6 hour period, was more complete, the total recovery as hippuric acid averaging about 61 per cent as compared with 45 per cent after the ingestion of the sodium benzyl succinate.

These results indicate that the findings of the *in vitro* experiments concerned with the hydrolysis of the esters of succinic acid by lipase (2, 4) do not hold in the living animal. If lipase were unable to hydrolyze salts of acid esters or the acid esters themselves, then no increases in the elimination of hippuric acid should have been observed after the ingestion of sodium benzyl succinate. If the cleavage of the esters of the dicarboxylic acids reaches an equilibrium when one of the carboxyl groups has been liberated, then the elimination of hippuric acid after the administration of dibenzyl succinate should correspond to less than 50 per cent of the benzoic acid, which could be derived from the benzyl groups of the ester. The more rapid excretion of hippuric acid after the administration of the soluble monobenzyl ester (corresponding to a more rapid hydrolysis and absorption) might have been anticipated, but no explanation is evident for the very low total recovery in the 24 hour period. Since benzyl alcohol has been shown by Snapper and coworkers (8) and in our own experiments to be oxidized rapidly to benzoic acid and excreted as hippuric acid, it is logical to believe that the results obtained with the esters are due to differences in the rate of hydrolysis and absorption from the intestine.

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SUMMARY.

1. After the oral administration of benzyl alcohol to rabbits, the velocity of hippuric acid excretion by the kidneys was only slightly less than that obtained after the feeding of sodium benzoate. This indicates that the oxidation of benzyl alcohol to benzoic acid is very rapid, being at least as rapid as the rate of synthesis and elimination of hippuric acid.

2. After oral administration of the sodium salt of monobenzyl succinate, more hippuric acid was eliminated in the 6 hour period immediately following the feeding than after oral administration of the dibenzyl ester of succinic acid, but the total amount recovered as hippuric acid in the entire 24 hour period was less than after feeding the dibenzyl ester.

3. These results indicate a hydrolysis of the monobenzyl ester of succinic acid *in vivo*, which is contrary to what might have been anticipated from studies of enzymatic hydrolysis *in vitro* (2–4).

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THE OCCURRENCE OF LABILE PHOSPHORUS IN VARIOUS KINDS OF MUSCLES.

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Since Engelmann's (1871) demonstration of increased phosphorus excretion in urine secreted during muscular exercise, there have been many attempts to show that the increase originated in the muscles. Muscle phosphorus compounds remain obscure, and it has only been during the last 10 years that the inorganic phosphorus of tissues could be conveniently determined.

During this period, tissue inorganic phosphorus has been determined in the extracts from acid protein precipitation, as for example trichloroacetic acid and the hydrochloric acid-bichloride mixtures. In such acid extracts from muscle, phosphorus was found which reacted with reagents for inorganic phosphorus. The study of this acid-soluble inorganic phosphorus fraction has been made particularly significant by Embden's lactacidogen theory, which assigned to phosphorus and lactic acid in muscle a common mother substance.

Although much effort has been expended to connect the facts of inorganic phosphorus and lactic acid metabolism in muscle, it is not easy to find positive evidence of the relation. The acid-soluble inorganic phosphorus is evidently a characteristic quantity for various types of muscles, and there is variation under some physiological conditions, but not uniformly in those where lactic acid is being rapidly produced.

That the conventional acid-soluble inorganic phosphorus does not represent the amount present in living muscle, was first announced by Eggleton and Eggleton (1927, *a*). When a modification of the Bell-Doisy method of phosphorus determination was used, the color development in ice-cold trichloroacetic acid

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filtrate from frog muscle was much slower than in the standard prepared simultaneously. By comparison of the rate of color development in standard and muscle extract it appeared that the initial inorganic phosphorus of frog striated muscle was only about 30 per cent of the ordinary value given by acid extraction at room temperature. This minimum, estimated from the curves by extrapolation to zero time, was greatly increased by fatigue so that one could believe that muscle exhaustion caused depletion of the labile phosphorus. A muscle in rigor had likewise lost its labile phosphorus, so that evidently the amount of labile compound is also related to the physiological condition of the muscle.

Recently, and apparently independently, Fiske and Subbarow (1927), presented a preliminary report on the results of similar findings in mammalian muscle. They used a colorimetric method of determination previously developed by them (1925) in which the color development is very rapid. Consequently, the estimation of such a labile compound is facilitated. The preliminary statement designates the labile phosphorus as phosphocreatine. Meyerhof (1927) reports also in a preliminary paper, the isolation of Fiske and Subbarow's phosphocreatine and conveniently incorporates its breakdown with the anaerobic energy-giving reactions in muscle.

If the final conclusions substantiate the indications of Fiske and Subbarow's preliminary statement, a new aspect of the part of inorganic phosphates in muscle metabolism is revealed. While Eggleton and Eggleton (1927, *b*) associate the appearance of labile phosphorus with decomposition of a phosphate carbohydrate, Fiske and Subbarow attribute its appearance to decomposition of a creatine compound. No reconciliation of these two views appears possible, and the first conflicts with the facts supporting Embden's lactacidogen theory.

The conventional method of acid extraction at room temperature does yield a phosphorus fraction having a normal value and capable of reproducible variation under certain experimental conditions. The entire evidence for the part of phosphorus in intermediary carbohydrate metabolism comes from a number of sources (yeast, blood, *etc.*) besides muscle, and is derived from data obtained by the old method of acid extraction at room temperature. Consequently, the value of the evidence for the conclu-

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sions would be invalidated if, as Eggleton and Eggleton (1927, *b*) believe, the source of labile phosphorus is a hexose monophosphate.

The acid-soluble inorganic phosphorus, as conventionally determined, is evidently the sum of the amount present in natural muscle and the labile phosphorus set free in acid at room temperature. It would seem strange if this variable system could furnish the basic evidence regarding another system so different as that postulated in the lactacidogen theory, but still possible if the sum of labile phosphorus and inorganic phosphorus is a definite quantity. This point has been reexamined and the results presented show that the probable phosphorus-carbohydrate relations are indicated by a different set of data from that which characterizes the labile phosphorus.

If the labile phosphorus originates in a phosphorus-creatine compound, its absence or deficiency would be expected in creatine-poor muscles. From the analytical data compiled by Hunter (1922) the creatine content of smooth muscle is from one-fifth to one-tenth as much as in the striated muscle of the same animals. These small creatine values have, furthermore, not been confirmed by isolation of creatine itself, so that there is room for skepticism as to their accuracy. But in phosphorus content smooth muscles are also lower than striated muscles in about the same ratio as the creatine values reported. Otherwise the work of Evans (1925) suggests that the essential carbohydrate transformations are similar in smooth and striated muscle, at least in so far as the utilization of glycogen and production of lactic acid serve as indicators.

In view of the interest of the facts observed, and their implications, striated muscle, heart muscle, and smooth muscle from a number of sources were examined. In the muscles examined the labile phosphorus was easily demonstrable in the striated muscle of mammals and turtles only. Bird muscle was not examined, and Eggleton and Eggleton had shown the labile compound in frogs. In seven quite different species of fish labile phosphorus was not demonstrated, nor was it present in insect striated muscle. Heart muscle and smooth muscle of vertebrates and invertebrates likewise gave no positive indication of the labile compound.

For determinations the procedure of Fiske and Subbarow (1925) was followed; the muscle was extracted (previously pow-

dered in liquid air) in ice-cold 8 per cent trichloroacetic acid—normally for 2 hours. Under the conditions observed the extraction for only a few minutes, reported satisfactory by Eggleton and Eggleton (1927, *a*) was not found adequate. No decrease of the labile compound occurred even after 5 hours at ice temperature.

In view of the suggestion that neutralization prevented decomposition of the compound, several neutral or alkaline protein precipitants were tried, but without success. The alkaline zinc hydroxide protein precipitant used in the Hagedorn-Jensen blood sugar method extracted no phosphorus whatsoever, and sodium tungstate reacted with the reagents for the phosphate determination.

Samples from the ice-cold filtrate were mixed with the reagents for Fiske and Subbarow's colorimetric method of determination

TABLE I.

Results of Colorimetric and Gravimetric Phosphorus Determination.

Phosphorus as mg. per gm.

	Guinea pig 1.						Guinea pig 2.			
Extraction time, *hrs.*	½	1	3	6	20*	20*	½	½	½	1
Colorimetric	0.70	0.91	0.94	0.83	0.88	0.91	0.62	0.77	0.77	0.85
Gravimetric	0.73	0.91	0.95	0.91	0.95	0.91	0.64	0.79	0.75	0.91

* Extraction by Schenck method.

and compared with standards having fully developed color. In this procedure the difference between the phosphorus concentration of unknown and standard includes a discrepancy due to the time necessary for color development even with Fiske and Subbarow's highly sensitive reagent. This fact, combined with the steeply rising character of the curve of phosphorus concentration against time during the first few minutes, and the rapidity with which the colorimetric matchings must be made, make the early position of the curve unreliable. To confirm this view a number of curves were made from observations on the phosphorus of muscle extracts which had been subsequently kept for 24 hours at room temperature. The difference between the curves for development of color in the ice-cold filtrates and those kept for a

day at room temperature represents the amount of labile phosphorus.

Some colorimetric determinations were made with a Duboscq type colorimeter. The majority and most satisfactory were with

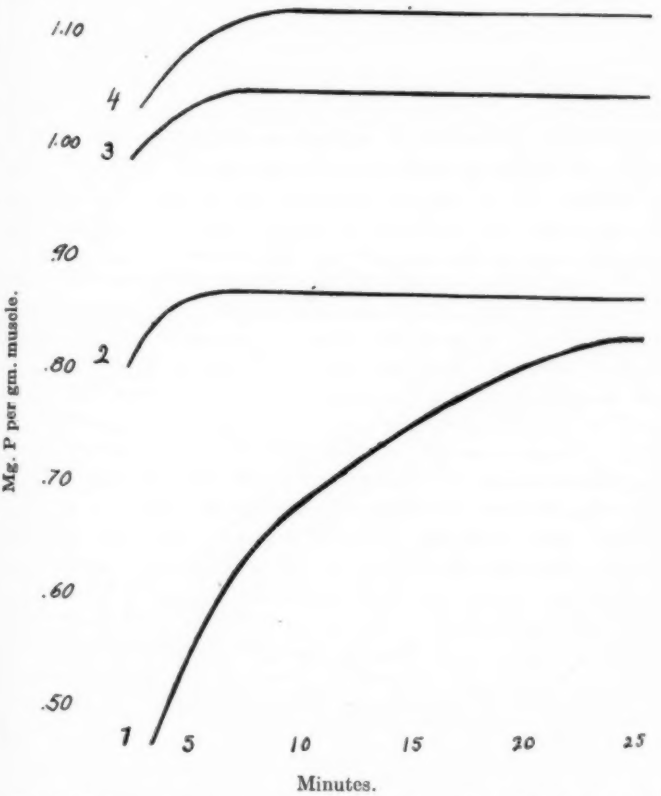

FIG. 1. Guinea pig gastrocnemius muscle extracted 3 hours in ice-cold 8 per cent CCl_3COOH. Curve 1, immediate; Curve 2, 24 hours; Curve 3, 48 hours; Curve 4, 72 hours.

a Leitz-Buerker colorimeter, in which the light passes through the same depth of standard and unknown solutions for each cylinder.

The results in Table I indicate the general reliability of the methods used. Evidently a maximum or, at least, a constant extract is secured in from 1 to 20 hours, but less than 1 hour is insufficient for the method.

Mammalian Muscle.

The labile phosphorus was easily demonstrated in one experiment on the gracilis muscle of a resting cat and in the hind leg muscles of the rat and of ten resting guinea pigs. In Fig. 1 typical curves are shown for the phosphorus (calculated as mg. of P per gm. of muscle) in guinea pig hind leg muscle. Curve 1 shows the increase in phosphorus concentration of the fresh muscle extract, in the colorimeter, with time. Curve 2 shows the results of similar determinations after the extract had stood for 24 hours at room temperature. Curves 3 and 4 indicate determinations on the same filtrate after 48 and 72 hours respectively.

The difference between the curves for muscles examined while the extract was still ice-cold and the curves for muscle extracts kept at room temperature is plain. It becomes more distinct in terms of per cent of full color reached at each time, as shown in Fig. 2. There is no significant difference between Curves 2, 3, and 4 by these methods of representation, and the type of Curve 1 shows the characteristic slope where labile phosphorus is present.

From the experiments made it would be indicated that the average minimum phosphorus of resting guinea pig muscle was about 40 per cent of the usual acid-soluble fraction, or that the labile part decomposed by acid amounted to 1½ times the amount already present. The absolute minimum is hard to determine, because the slope of the curve is steep and the error introduced by the observer in the first few hasty colorimetric readings may be quite large. All of the indications suggest that the actual minimum for inorganic phosphorus in the resting muscles is probably less than 40 per cent of the acid-soluble fraction.

In the single case of rat muscle examined the minimum value was 0.35 mg. of P per gm. of muscle, with a maximum of 0.80. The single cat gracilis muscle examined had a minimum of 0.25 and the unusually low maximum, for these animals, of 0.75.

Muscles from about the pectoral girdle of the turtle had 0.40 and 0.25 as minima, and 0.78 and 0.64 as maxima, indicating about

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50 per cent as labile phosphorus in each case. These values may not represent the minimum attainable because of delay and injury incidental to the removal of the shell. It is significant principally that the proportion of labile phosphorus is quite similar in turtles and mammals. In two of the series of guinea pig muscles (Table II) examined the amount of labile phosphorus extracted in different times was considered. While, on account

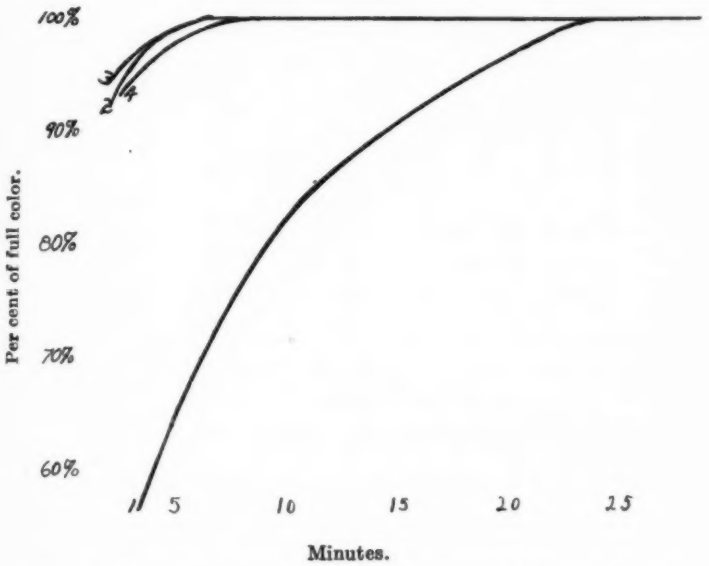

FIG. 2. Guinea pig gastrocnemius muscle extracted 3 hours in ice-cold 8 per cent CCl_3COOH. Per cent of maximum P. Curve 1, immediate; Curve 2, 24 hours; Curve 3, 48 hours; Curve 4, 72 hours.

of the uncertainties of the method mentioned, there is considerable variation in the minimum, still the per cent found as labile phosphorus is not perceptibly different for short as compared with long extraction. Unless this results from decomposition during preparation and determination, it is indicated that the velocity of extraction is the same for both labile phosphorus and preexisting inorganic phosphorus.

Fish Muscle.

Fish muscle was first examined from two salt water fish, the midshipman (*Porichthys notatus*) and the hagfish (*Bdellostoma*

TABLE II.

Amount of Labile and Inorganic Phosphorus in Guinea Pig Muscle after Different Periods of Extraction.

Phosphorus as mg. per gm.

Guinea pig No.	Extraction time.	Minimum.	Maximum.	Labile phosphorus.
				per cent
C VII	30 min.	25	64	60
	1 hr.	25	80	68
	3 hrs.	35	80	55
	6 "	27	66	60
C IV	5 min.	22	58	62
	15 "	30	60	50
	30 "	25	60	59
	1 hr.	25	65	62
	4 hrs.	32	72	56

TABLE III.

Phosphorus Content of Fish Muscle.

Phosphorus as mg. per gm.

Source.	Minimum.	Maximum.	Difference in per cent of maximum.	Time for reaching maximum.
				min.
Squawfish, *Ptychocheilus*	1.37	1.45	6	6
Catfish, *Ameiurus nebulosus*	0.81	0.91	11	12
Black bass, *Micropterus salmoides*	1.33	1.50	11	7
Brook trout, *Salvelinus fontinalis*	0.99	1.18	16	11
Goldfish	0.90	1.02	10	8
Midshipman, *Porichthys notatus*	0.81	1.02	21	7
Hagfish, *Bdellostoma stouti*	0.92	1.01	9	7

stouti). If any labile phosphorus is present, it is only in insignificant amounts, in comparison with that of mammals. These fish had been living apparently normally for some time in aquaria,

but it was possible that they were still abnormal. Fresh goldfish muscle was, therefore, examined, but again without positively showing any labile phosphorus. As there was still a chance that transportation might have injured the goldfish, we secured, by the kind assistance of Dr. Evermann and Mr. Seale, four other kinds of fish from the Steinhart aquarium, San Francisco. The

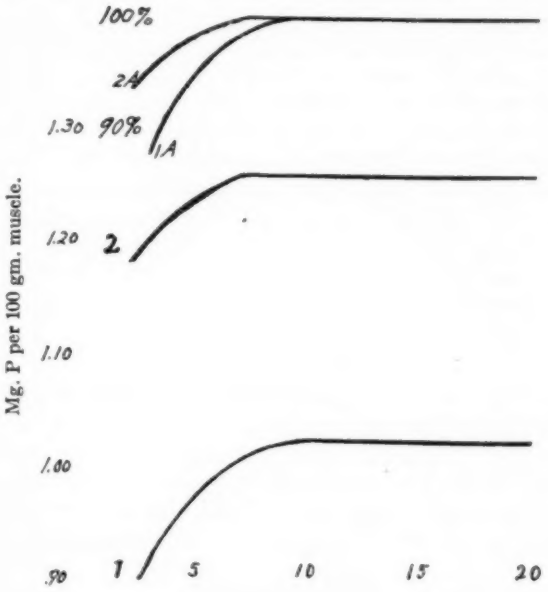

FIG. 3. Goldfish side muscle extracted 2 hours, 45 minutes in ice-cold 8 per cent CCl_3COOH. Curve 1, immediate; Curve 2, 24 hours later; A, per cent of maximum P.

fish were quickly removed from their tanks, killed, and pieces were cut from the side muscle and placed in liquid air. In this frozen condition the muscles were then transported to the laboratory for the actual determinations. None showed definitely any labile phosphorus.

These different fish included five fresh water and two salt water

species, quite distinct and representing a wide range of type in structure and habit. The figures in Table III show in each case that the minimum value, compared with the standard, is slightly low initially. Fig. 3 shows how the curve for fresh extract com-

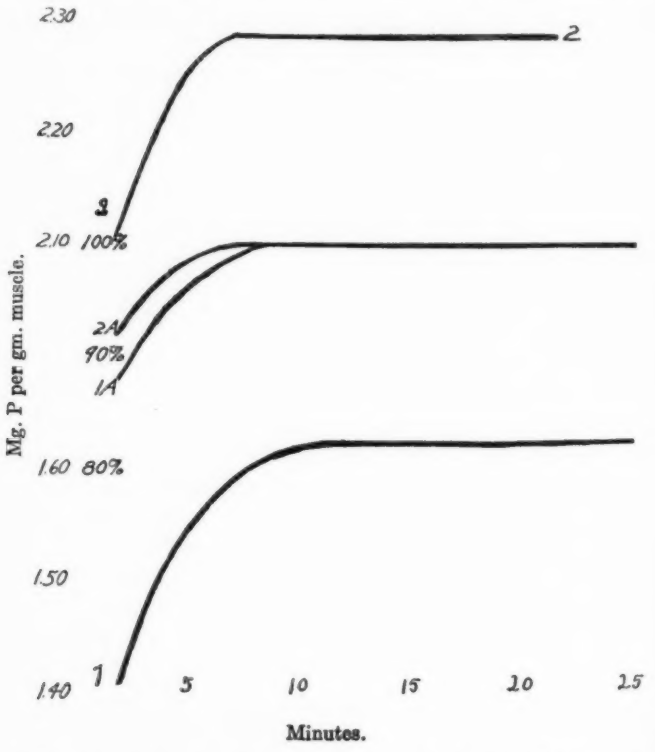

FIG. 4. Muscles of twenty-five grasshoppers' legs extracted 2 hours, 45 minutes in ice-cold 8 per cent CCl_3COOH. Curve 1, immediate; Curve 2, 48 hours later; A, per cent of maximum P.

pares with that for old extract. There is a small difference between the two such as might arise from the presence of labile phosphorus. But the possible amount would hardly equal 10 per cent of the acid-soluble inorganic phosphorus, and the maxi-

mum for the curve is reached in less than 10 minutes, while for mammalian muscle the maximum is not reached before 20 minutes. On account of the uncertainty of results in the steep part of the curve, it is safest to conclude that the labile phosphorus is not proved to exist in fish muscles, and that they are essentially different in this respect from the muscles of the other vertebrates.

The creatine content of fish muscle generally is quite of the same order as that of mammalian skeletal muscle (Hunter, 1922), so that if the phosphocreatine is characteristic of mammalian muscle the constituents, at least, are present in fish. But fish muscle is also different in the slowness of its postmortal glycogen decomposition compared with mammalian muscle (Macleod and Simpson, 1927), so that the similarity of fish to other vertebrate muscle may not be very close. It is unfortunate that no trials of elasmobranch muscle could be made, for Hunter (1922) remarks that the meager data on their creatine-creatinine excretion show that, like mammals, they excrete principally creatinine, while the teleosts are like birds in excreting principally creatine.

Invertebrate Striated Muscle.

If labile phosphorus is generally associated with rapid contraction, it might be expected to be present, particularly in the striated muscles of insects. The leg muscles from twenty-five grasshoppers were, however, like fish muscle in not showing the certain presence of labile phosphorus (see Fig. 4). The labile compound, if present at all, is insignificant in amount compared with that of mammals.

Invertebrate Muscle.

The same condition appears in the muscles of the crab's chela, which showed no positive indication of labile phosphorus.

The pallial muscle of the large clam, *Schizothaerus nuttallii*, was examined, and in Fig. 5 are shown results of several determinations of phosphorus fractions.

The lowest curve shows a small inorganic phosphorus content immediately in cold trichloroacetic acid extract. There is no perceptible increase with time in the colorimeter so that the absence of truly labile phosphorus is certain.

The next curves show that the Schenck method of extraction

gives results agreeing with those on the day old trichloroacetic acid extract. These colorimetric determinations were confirmed by Embden's (1921) gravimetric method. No labile phosphorus, according to its definition, would be expected in the day old extracts, but the amounts are significantly greater than those derived from cold extract. Consequently there has been some decomposi-

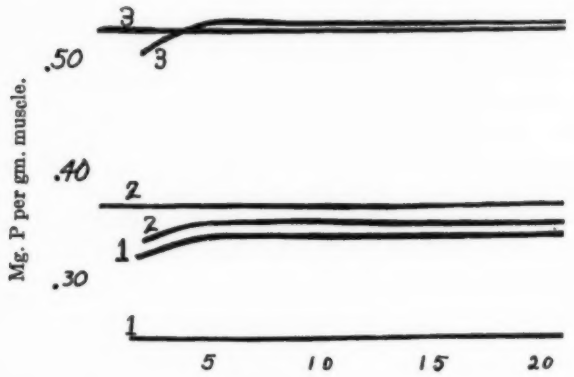

FIG. 5. Pallial muscle of *Schizothaerus nuttallii*. Curves 1, clam muscle extracted 2 hours in ice-cold 8 per cent CCl_3COOH. The lower Curve 1 represents determination by the colorimetric method immediately; the upper Curve 1, determination by the same method 24 hours later. Curves 2, clam muscle extracted 15 hours in acid $HgCl_2$ solution (Schenck). The lower Curve 2 represents determination by the colorimetric method; the upper Curve 2, determination by the gravimetric method. Curves 3, clam muscle digested 2 hours in 2 per cent $NaHCO_3$ solution (Schenck). The lower Curve 3 represents determination by the gravimetric method; the upper Curve 3, by the colorimetric method.

tion of organic phosphates in the extract. The decomposition occurred exclusively in the extract because the trichloroacetic extract which had remained only 2 hours in contact with the muscle was used in one case and gave results practically agreeing with the Schenck extract, which had been for 15 hours in contact with the muscle.

Not all of the organic phosphorus was released in the acid extraction, because incubation of the muscle at 40° for 2 hours in 2 per cent sodium bicarbonate caused a considerable increase in inorganic phosphorus. This last method, following Embden's directions, gives by its excess over the acid-soluble inorganic phosphorus the magnitude of the lactacidogen content. Judged from the figures, there is an appreciable lactacidogen content. If not absolutely, at least by comparison with the

TABLE IV.

Phosphorus Content of Smooth Muscle.

Phosphorus as mg. per gm.

Source.	Minimum.	Maximum.	Time for reaching maximum.
			min.
Bladder.			
Guinea pig	0.21	0.31	9
Two guinea pigs	0.135	0.16	12
Turtle	0.07	0.082	11
Intestine.			
Rabbit	0.23	0.26	8
Two rats	0.21	0.22	6
Turtle	0.22	0.27	11
"	0.15	0.17	8
Uterus, virgin.			
Guinea pig	0.14	0.15	8
" "	0.23	0.23	
Three rats	0.21	0.22	11

amount of inorganic phosphorus originally present, this is similar to the lactacidogen content of vertebrate striated muscle. There is, however, the difficulty of determining which acid extract to take as the initial value. But in either case, the clam smooth muscle is similar to vertebrate striated muscle in having an appreciable amount of inorganic phosphorus released by incubation with bicarbonate. In lacking the true labile phosphorus it differs. Evidently the labile phosphorus is not the sole source of muscle inorganic phosphorus.

Smooth Muscle of Vertebrates.

The examination of smooth muscle from the organs of several vertebrates did not positively show any labile phosphorus present. (This fact has been announced by Eggleton and Eggleton (1927, *b*) and was first noticed since our work was completed.) In the results quoted in Table IV are given the minimum and maximum of phosphorus values observed. It is evident that muscle from the intestine of rabbits, rats, and turtles, and from the virgin uterus of the guinea pig and rat possesses no labile phosphorus. Extracts from bladder muscle of the guinea pig and turtle show a change in the colorimeter so that they may contain a small amount of labile phosphorus. Otherwise, the inorganic phosphorus of

TABLE V.

Phosphorus Content of Heart Muscle.

Phosphorus as mg. per gm.

Source.	Minimum.	Maximum.	Time for reaching maximum.
			min.
Two guinea pigs	0.30	0.33	8
Rabbit	0.40	0.44	9
Three rats	0.41	0.43	10
Turtle	0.14	0.16	15
"	0.14	0.17	8

bladder muscle is similar in amount to that found in other smooth muscle.

Smooth muscle generally contains lactacidogen as determined by increased inorganic phosphorus after incubation (Evans, 1925), although in small amounts only. Its acid-soluble inorganic phosphorus as shown in Table IV, is only about $\frac{1}{5}$ that found in skeletal muscle generally. More significant in the light of Fiske and Subbarow's work is the relation between the lack of labile phosphorus and the lack of creatine. According to Hunter (1922) one may be quite skeptical as to the actual presence of creatine in smooth muscle, although it has been reported often, for the reports are not based upon actual isolation of the substance. Riesser (1922) believed that he could establish a relation between the

creatine content and lactacidogen of muscles, showing that heart muscle contained only small amounts of each, while uterus muscle contained even less. The parallelism appears in his figures, but it does not suggest that the lactacidogen and labile phosphorus are identical, although they may well concern each other.

Heart Muscle.

In heart muscle we can hardly agree with Eggleton and Eggleton (1927, *b*) in finding the labile phosphorus equal to $\frac{1}{5}$ of that usually present in skeletal muscle. Table V shows a slight gradual increase in color when compared with a fully developed standard. This

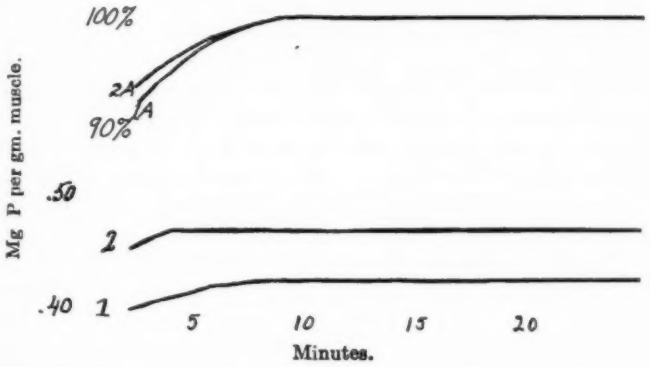

FIG. 6. Two guinea pig hearts extracted 3 hours, 45 minutes in ice-cold 8 per cent CCl_3COOH. Curve 1, immediate; Curve 2, 24 hours later; A, per cent of maximum P.

increase is so small as to be of uncertain significance in comparison with the slight delay in color development even in the standard. A comparison of the curve for a still cold extract and one kept for 24 hours at room temperature (Fig. 6) shows a slight lag for the fresh preparation, but no significant difference. The necessary conclusion appears to be that the labile phosphorus, defined as it is by rapid increase in phosphorus in acid solution, is lacking in heart muscle.

DISCUSSION.

The nature of the origin of labile phosphorus in muscle has not yet been established and we have only preliminary reports to indicate the nature of its precursor. Judged by the definition as a substance yielding acid-soluble inorganic phosphorus in acid solution, it is undoubtedly present in the skeletal muscle of vertebrates except fish. It is distinctly different from Embden's lactacidogen, in that by definition the latter yields acid-soluble inorganic phosphorus in weakly alkaline solutions. Furthermore, in acid solution the labile phosphorus is practically completely decomposed within a half hour, after which time the acid-soluble inorganic phosphorus content of the extract remains constant for about a day before showing further increase.

The name "phosphagen" suggested by Eggleton and Eggleton (1927, *a*) and adopted by Meyerhof (1927) is, therefore, a contradiction of the facts, because the labile phosphorus indicated is only one source of inorganic phosphorus in muscle.

Whether or not the labile phosphorus exists in the muscle before the process of extraction, it has definite physiological significance because of its regular variation under different physiological conditions.

Although the labile phosphorus as found in mammalian skeletal muscle is lacking in fish and insect striated muscle, in invertebrate muscle, smooth muscle of vertebrates, and in heart muscle, its function may be assumed by similar substances which are not, however, preserved by the methods of study. Consequently, we can only deny its presence in the strict terms of the original definition. Until the definitive announcements of the nature of labile phosphorus present their evidence, the suggested importance of their function should not be clouded by rash speculation.

CONCLUSIONS.

Labile phosphorus is easily demonstrated in skeletal muscle of mammals according to the methods defined by its discoverers.

It is distinct from Embden's lactacidogen both by definition of the conditions for determination and by the facts of its appearance. Lactacidogen occurs in smooth muscles entirely lacking labile phosphorus.

XUM

Labile phosphorus could not be demonstrated in fish striated muscle, grasshopper striated muscle, smooth muscle generally of vertebrates and invertebrates, and heart muscle. If possibly present in any of these muscles, its amount is of quite a different order from that in mammalian skeletal muscle.

The occurrence and lack of labile phosphorus follow, in some respects, the occurrence of creatine, a natural consequence if Fiske and Subbarow's announcement of the labile compound as a phosphocreatine is substantiated.

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STUDIES ON THE EFFECT OF TEMPERATURE ON THE CATALASE REACTION.

VI. HEAT INACTIVATION OF CATALASE AT DIFFERENT HYDROGEN ION CONCENTRATIONS.

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In a previous paper (1) we presented preliminary data on the inactivation of catalase at different temperatures. This study has been extended to determine the effect of variations in H ion concentration on the rate and degree of inactivation by heat, the results of which are now reported.

Methods.

The method of experimentation in the present investigation has been improved in several essential points as compared to the original procedure. To avoid the undeterminable loss of activity incident to the warming of the enzyme solution to the desired temperature, a definite amount of a buffer mixture of known pH was heated to about 2° above the desired temperature. A measured quantity of enzyme was then introduced, this quantity being selected to give the proper enzyme concentration in the mixture. The addition of the enzyme solution at the same time cooled the buffer-enzyme mixture, reducing its temperature practically to the desired level. By preliminary tests experience was gained whereby the adjustment of the final temperature upon addition of the enzyme to the buffer has been successful to within 0.1–0.2° of the temperature aimed at. The temperature of the mixture was measured with a Bureau of Standards thermometer inserted in the flask. A sample of the buffer-enzyme mixture was measured out at once, serving for the 0 time determination.

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With practice, these several operations were carried out very rapidly, practically within very few seconds. The buffer-enzyme mixture was now placed in a thermostat maintained automatically at the experimental temperature. Further samples were removed at set intervals. The measured samples were at once transferred to the bottle in which the catalase reaction was finally determined, the samples being cooled by dilution with the contents of the bottle. These bottles contained the required amount of water strongly buffered for pH 7.0 and were placed in the large shaking machine-thermostat already described (2) set for 21°. This temperature was selected because of the simple correction that is necessary for the destruction of enzyme during the reaction, exactly one-half of the maximum enzyme activity prevailing at 2° (2). This correction was made use of in calculating the units of catalase lost through heating at various temperatures and H ion concentrations for various lengths of time. At least two separate determinations of catalase activity were made under the same experimental conditions, different quantities of the enzyme, inactivated at any one of the different temperatures, being used. This provided at least two experimentally determined points by means of which it was possible to construct the corresponding catalase isotherm (see Paper V, (2) p. 94) and from this to find the number of catalase units required to decompose 70 per cent of a definite amount of hydrogen peroxide. The degree of inactivation resulting from exposure to high temperatures at different pH was easily calculated from these data. The concentration of the catalase solution was carefully standardized, a definite volume containing 1 enzyme unit at 2° (or 0.5 units at 21°). If, for instance, following an exposure at 60° and pH 6.0, for 5 minutes, an amount of enzyme solution equivalent to 2.15 units of the unheated catalase preparation were now required to produce the same effect (70 per cent decomposition of the hydrogen peroxide) 46.5 per cent of the original catalase activity remained, whereas, if, under the same conditions but at pH 8.0, the equivalent of 3.65 units were required, only 27.4 per cent of the catalase activity was still present. Thus, all comparisons were made on a strictly quantitative basis, a definite amount of enzymatic work being used as the criterion, which, of course, is the only theoretically correct basis to use.

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It may not be amiss in this connection to say a word with regard to changes in H ion concentration of buffers at high temperatures. We assume that the pH at which the inactivation is accomplished is practically the same as that determined from the ratio of the buffer components at ordinary room temperature. It is, of course, a well recognized fact that the pH of buffers is affected more or less by high temperatures (3, 4). However, the extent of the deviation depends very much upon the nature of the buffer mixture and on the pH range. Thus, it was shown by Walbum (4) that borate buffers are least affected by a rise in temperature and even then the greatest change occurs on the alkaline side. As the temperature of a borate buffer of pH 9.23 at 20° was raised to 70° it became 0.37 of a pH more acid, but the nearer to the neutral point the buffer mixture was the less marked was the alteration of its pH at 70°. In our experiments Kolthoff's series of buffers (borate-phosphate mixtures) were used, and, since the temperature did not exceed 65°, we are justified in assuming that the pH of the mixtures in which the inactivation of the catalase took place was not materially different from that designated.

Degree of Inactivation.

The inactivation of catalase at temperatures up to 40° is insignificant and we commenced the series of experiments at 50°. Even at this temperature inactivation proceeds very slowly, except at certain H ion concentrations. Thus, at pH 4.0 the catalase is quickly and completely inactivated, but at pH 4.5 there is already a well marked change, about 10 per cent of the catalase activity still remaining even after 30 minutes heating at 50°. With further increase in the pH of the medium in which inactivation takes place the effect of heating becomes less and less pronounced. The least inactivation occurs at pH 6.0, but as the medium becomes alkaline the rate of inactivation increases once more and is as great at pH 8.0 as it was at pH 5.0. The inactivation on the alkaline side, however, is in marked contrast to that on the acid side, since it is considerably slower and less extensive. At the pH 6.0 to 7.0, where least inactivation is effected, another interesting fact appears; namely, that the inactivation proceeds more or less rapidly at first but soon slows down, attaining an

equilibrium before the inactivation by heat is completed. At such pH even 4 to 6 hours heating at 50° has no appreciable influence on the activity of the remaining enzyme. In this instance equilibrium is reached when approximately 70 to 75 per cent of the catalase is still active.

At higher temperatures inactivation proceeds with much greater speed and the pH zone, within which catalase activity, at least temporarily, persists, becomes more contracted. This effect of H ion concentration is especially pronounced on the acid side of pH 6.0. Thus, at 57.5° catalase is immediately inactivated at H ion concentrations below pH 5.0, and even at pH 5.0 the inactivation is so rapid that only a trace of the original catalase activity remains after 25 minutes exposure (about 2 per cent residual activity). The least inactivation at this temperature occurs again at pH 6.0 or even at pH 6.5, where after 4 hours heating there is still 9 per cent residual activity. At the opposite end of the pH range the inactivation is immediate at pH 9.0, while at pH 8.0 the inactivation is also rapid but there is about 7 per cent of residual activity after heating for 1 hour. At the optimum H ion concentration, *i.e.* pH 6.0, the inactivation rate decreases very much after the first 2 hours of heating, but there is no definite equilibrium state as was the case at 50°. Thus, after 2 hours heating at 57.5° and pH 6.0 as much as 73 per cent of the catalase activity was lost, while during the 3rd hour only 10 per cent more of the catalase disappeared.

When the heating is carried out at still higher temperatures, the rate of inactivation increases, while the pH zone, where the loss of activity is not instantaneous, becomes progressively narrower. Thus, at 60° the catalase became immediately inactivated at pH 5.0 and at 63° at pH 5.5. On the alkaline side, the inactivation does not proceed quite so rapidly and at pH 8.0 some activity is retained even at 60° but is completely lost at 62°. Even at pH 7.5 after heating at 62° there is very little residual activity. At 63° and 64° some catalase activity is retained within a H ion concentration range of pH 6.0 to 7.5, being completely lost within 5 minutes of heating at pH 7.0 to 7.5. At 65° the catalase is completely and instantly inactivated at all H ion concentrations. We may, therefore, regard 65° as the critical temperature for catalase activity.

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The degree of inactivation of catalase at different temperatures and pH at 0 time and at intervals of 5 and 10 minutes of heating, respectively, is shown in the series of curves in Figs.

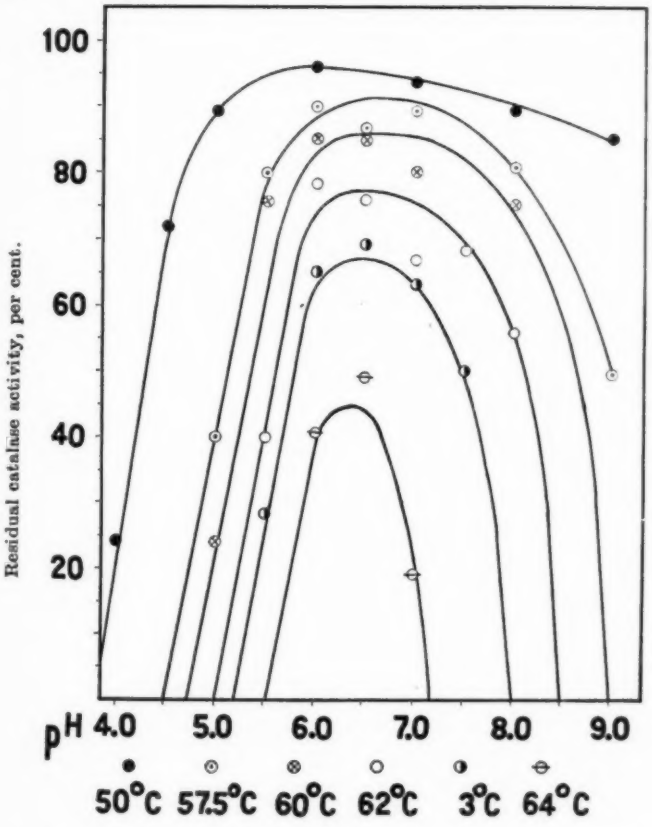

FIG. 1. The solid line curves in Figs. 1, 2, and 3 represent the changes in relative *activity* of a beef kidney catalase preparation at various temperatures and H ion concentrations at 0, 5, and 10 minutes exposure. The dotted line curves represent the changes in the catalase *reaction* at corresponding pH. All curves are drawn to the same scale, and the same symbol is used throughout to indicate each temperature.

1 to 3. It can be seen from these, first, that the inactivation of catalase at H ion concentrations below pH 5.5 proceeds very abruptly, whereas on the alkaline side the rate of inactivation, with increasing temperature and alkalinity, changes more gradu-

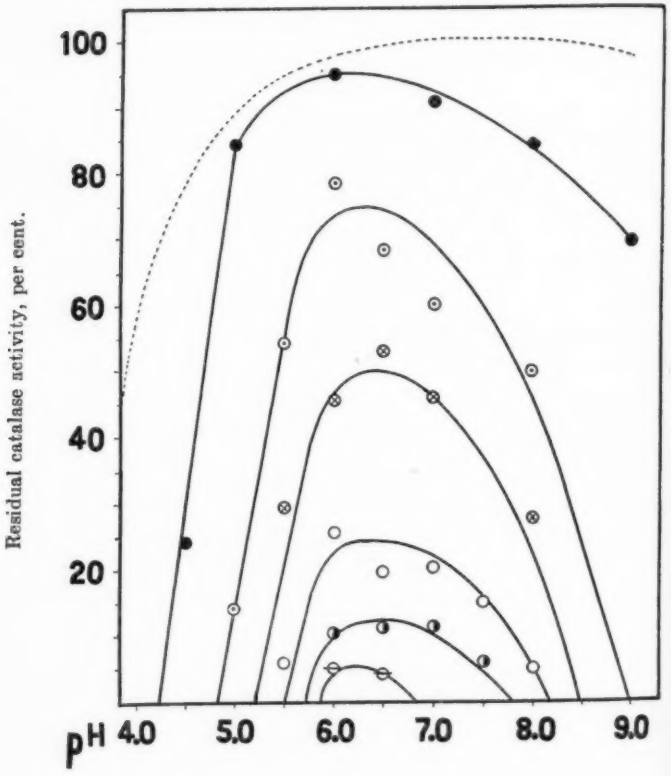

FIG. 2.

ally. Secondly, that at pH 6.0 to 6.5 catalase has the greatest stability to heat, except at 65°, at which temperature there is immediate inactivation irrespective of the reaction of the medium.

It is particularly interesting to point out in this connection that heating catalase at 50° within the range of pH 6.0 to 7.0 does not

result in complete loss of its activity, 70 to 75 per cent remaining unaffected even after 6 hours exposure to this temperature. This residual activity is greater the lower the temperature to which the catalase is heated. At higher temperatures the inactivation

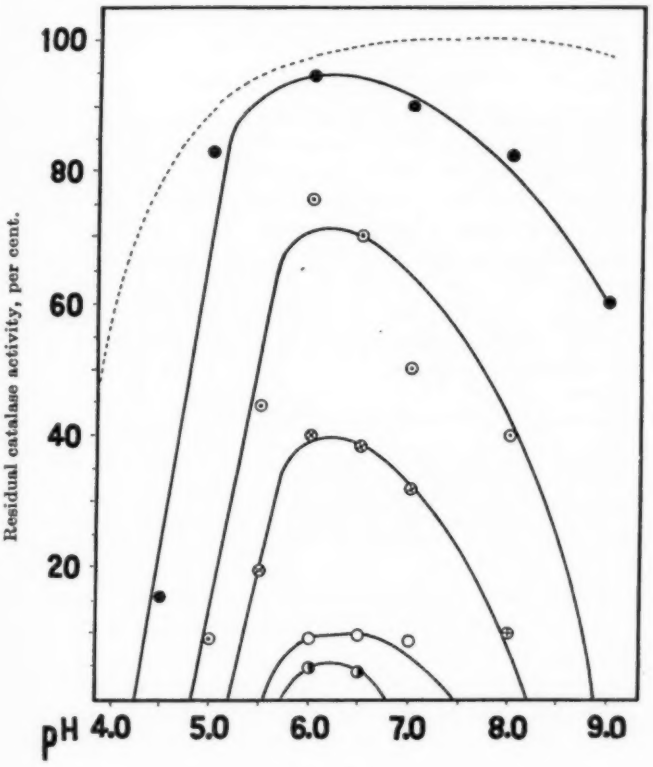

FIG. 3.

proceeds to completion except that at 57.5° 20 per cent of the activity still remains intact after 5 hours of heating at the pH of 6.0. We are, therefore, led to conclude that the heat inactivation must be an equilibrium reaction which may be represented

symbolically thus, $E_M + [H^+] \rightleftarrows E_{MI}$, where E_{MI} is the inactivated material. The position of the equilibrium is determined by the H ion concentration, and the rate of change by the temperature. We shall see presently that the heat inactivation of catalase is a process which can be definitely differentiated from the destruction of catalase by hydrogen peroxide which likewise varies with temperature.

Course of Inactivation Reaction.

The rate of heat inactivation of catalase at various temperatures and H ion concentrations follows very closely the bimolecular equation. At any rate, within certain ranges of combination of those two factors the constants calculated by means of this equation are generally in good agreement. The heat inactivation of catalase is quite distinct in this respect from its destruction by peroxide ($E_A \rightarrow E_O$) which was shown to be a monomolecular reaction (1). Shaklee (5) has shown that pepsin becomes inactivated at 37°, this inactivation likewise following the course of the bimolecular reaction.

Latent Period of Reaction.

The latent period of the catalase reaction depends directly upon the degree of inactivation of the enzyme, becoming very extensive when this has been exposed to high temperatures or to acidities of pH 4.0 and below that. In an earlier paper (6) it was suggested that the latent period is the time required for the accumulation of a sufficient concentration of active catalase, $E_M \rightarrow E_A$, to initiate the reaction, the rate of accumulation being proportional to the concentration of E_M. In Table I are recorded the latent periods observed in experiments with catalase inactivated by heat at varying H ion concentrations and for varying lengths of time. A survey of the data presented in this table indicates the direct relationship between the degree of inactivation and duration of the latent period. It will be further noted that the latent period increases much more on the acid than on the alkaline side of the optimum H ion concentration (pH 6.0 to 6.5). At this pH of maximum resistance of the catalase to heat the latent period is lengthened either after very prolonged exposure to heat or after exposure to heat of 60° and above. That the

latency of reaction actually depends upon the concentration of E_M is borne out by the fact that, if enough inactivated material is used to accomplish about 70 per cent decomposition of the hydrogen peroxide, the latent periods are practically the same as those observed in enzyme solutions heated at pH 6.0 to 7.0.

DISCUSSION AND SUMMARY.

In this paper the results of an investigation are presented on the effect of temperature on *catalase*, its effect on the catalase *reaction* having been the subject of previous contributions to this series. The enzyme is very slowly inactivated at temperatures below 50°, the inactivation becoming very rapid as the temperature is raised to 55° or above, especially at pH less than 6.0. But it is somewhat slower at pH more than 7.0. At pH 4.5 the inactivation is instantaneous at temperatures above 50°, while at pH 8.0 this does not occur until 63° is reached. At 65° the enzyme is instantly inactivated independently of the H ion concentration. In other words, 65° is its critical temperature.

Under proper experimental conditions the heat inactivation of catalase is an equilibrium reaction and under those conditions there is practically no change in the residual activity with increasing time. The point to which inactivation proceeds is determined by the degree of temperature and by the pH of the medium. The rate of the heat inactivation follows that of a bimolecular reaction.

Von Euler (7) and Lüers and Lorinser (8) have found that sucrase and amylase, respectively, show a maximum heat stability (they did not experiment with temperatures above 55°) at a H ion concentration which coincides with that required for optimum enzymatic activity. In other words, these enzymes are most stable in the form in which they are most active, which is obviously not true for catalase, where the maximum stability at pH 6.0 is in marked contrast to its rather extensive pH range of optimum activity.

This is evident from Figs. 2 and 3 in which the pH curve of the *catalase reaction* has been plotted as a dotted line to show its relation to the heat *inactivation of catalase* at corresponding pH. Careful examination of the type of curve of the effect of pH on the catalase *reaction* and of the effect of pH on the heat *inactivation*

TABLE I.

Latent Period of Catalase.

Temperature.	Time of heating.	Inactivation at pH:								
		4.0	4.5	5.0	5.5	6.0	6.5	7.0	8.0	9.0
°C.	min.	sec.	sec.	sec.	sec.	sec.	sec.	sec.	sec.	sec.
50	0	90	30	20		20		15	20	25
	5		65							31
	15		196						25	42
	30		320	30		20		12	28	70
	60		680	30		20		12	33	
	120			36		22		25	28	
	180			43						110
	240					(37)		(19)	48	
	360					28		29		
57.5	0			60	21	20		19	65	
	10			240	56			37	80	
	20			1800	70	20				
	30			2100	85	27	84	77	345	
	60			4200	127	15	115	130		
	90				222		145	525		
	120						228	(462)		
	180						360	917		
	240						(360)	2040		
60	0				43	18	17	21	148	
	5				104	42	48	31	220	
	10				645	86			310	
	20					218	121	148		
	30							185		
	45						(480)	(3600)		
	60					(600)				
62	0				38	24	22	42		
	5				(720)	172	131	274		
	10					496	446	(720)		
	20					1416				
	30					1614				
	45					(3000)				
63	0					26	27	23		
	5					567	280	340		
	10						1500			
64	0					30	30			
	5					(1800)	(900)			

Figures in parentheses are more or less approximate.

of catalase shows the marked difference between the two. It will be seen that there is no definite optimum H ion concentration for the *reaction*, which proceeds equally well over a range of pH 6.5 to 8.5, and is almost at its maximum even over a wider range, pH 6.0 to 9.0. There is, on the other hand, a point of maximum stability on the *inactivation* curves. The *reaction* curve likewise slopes off rapidly on the acid side but to a much less extent than is the case in the *inactivation* curves.

We have propounded the view (6) that catalase behaves like a weak acid, the anions of which alone possess catalytic activity. It was anticipated that in the heat inactivation experiments further corroboration of this conception would be found. But the distinct limitation of maximum stability to a definite pH necessitates a revision of our former view and a return to the older conception of the amphoteric nature of catalase.

It is necessary to assume also that catalase in the form of cations is inactive and, furthermore, that the reaction whereby the combination, in which catalase plays the part of a cation, is formed, is irreversible, since catalase exposed to the action of H ion concentration of the magnitude of pH 3.5 to 4.0 fails to regain its function under a favorable pH. On the other hand, the catalytic activity is shared by both molecules and anions, the latter really being heat-labile. We have already come to the conclusion from our previous studies that the anion is destroyed through oxidation by hydrogen peroxide, which process increases with alkalinity.

The idea that catalase is an ampholyte with an isoelectric point somewhere between pH 4.0 and 4.5 must be definitely discarded as erroneous as it has been derived from a limited study of the catalase *reaction* at different pH. The study of the heat *inactivation* leads one to postulate that catalase must have its isoelectric point near pH 6.0. It is obvious, of course, that it must possess stronger basic than acid properties inasmuch as it was shown before (6) that catalase has an apparent acid dissociation constant of an approximate magnitude of 1×10^{-4}. The anion is its most vulnerable part, as was observed in the study of the catalase reaction in alkaline media at different temperatures and as is corroborated now by the study of its heat lability.

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THE SPECIFIC DYNAMIC ACTION AND NITROGEN ELIMINATION FOLLOWING INTRAVENOUS ADMINISTRATION OF VARIOUS AMINO ACIDS.

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The term specific dynamic action, first introduced by Rubner, signifies an increase in total heat production following the administration of any of the primary foodstuffs or certain of their cleavage products. In spite of the large amount of work which has been done on the subject, relatively little is known concerning the fundamental cause of this increase in heat production. It has been quite definitely shown that it is not due to the increased peristalsis which is set up in the gastrointestinal tract by the taking of food, nor is it in the main due to the increased activity of the various glands whose primary function is the preparation of food for digestion and absorption.

The classic investigations of Lusk and his coworkers form the foundation of modern knowledge of the specific dynamic action of the various amino acids, and their data and conclusions must be taken as the starting point for all future investigations. On the basis of his work Lusk formulated his well known theory of amino acid stimulation which he defines as follows: "In amino-acid stimulation some oxy or keto acids derived from protein metabolism, stimulate the cells to a higher level of oxidative activity" (13). In practically all of his work the amino acids were administered by mouth and the determination of heat production was begun 1 hour after ingestion. Under these conditions the maximal increase in heat production was usually observed during the 2nd and 3rd hours after ingestion.

Oral administration is in certain respects the most ideal pro-

cedure from a purely physiologic view-point, but since Levene and Meyer (9) have shown that apparently there are differences in the rate of absorption of different amino acids from the gastrointestinal tract, it is possible that the response of the organism may be greatly altered and give rise to erroneous conclusions regarding the relative increase in heat production caused by the various amino acids. It was thought best, therefore, in these experiments, to adhere to intravenous administration in order that a known quantity of amino acid might be introduced into the body in a known period of time. The purpose of these experiments was twofold: first, to study the increase in heat production caused by various amino acids when administered intravenously to normal animals; and second, to secure data on normal animals which could be compared with the same procedure in the hepatectomized animal. In normal animals the nature of the response has been identical in all satisfactory experiments; the results obtained with alanine, glycocoll, and phenylalanine will be reported in detail.

Methods.

The animals were carefully trained before being used in actual experiments. During the training period the basal heat production was determined almost daily for a period of several weeks, and, in some cases, for several months. In satisfactorily trained animals the maximal variation in heat production, as determined by successive tests of 10 minutes duration, was usually within 1 calorie each hour over a period of 4 hours or more.

In most experiments 0.1 gm. of amino acid nitrogen for each kilo of body weight was the standard dose. This amount of amino acid was dissolved in from 50 to 70 cc. of distilled water or physiologic sodium chloride solution. Exceptions to this will be mentioned later. The solution was warmed to body temperature and injected into the saphenous vein; the time required for injection varied from 10 minutes to 1 hour and 11 minutes.

The method of indirect calorimetry employed was described in detail by Boothby and Sandiford (4) and adapted to animal use by Kitchen (7). Briefly the method consists in the collection of the expired air in a gasometer for periods of 10 or 15 minutes.

Duplicate analysis of aliquot samples is then made for the percentage of carbon dioxide and oxygen by use of the Haldane gas analysis apparatus.

The environmental temperature was maintained at about 25° by means of a large wooden box which was placed over the table on which the animal was lying. A large electric light bulb, placed near the top of the box, supplied heat when needed, while cooling the interior was effected by raising the hinged glass side of the box. Movements of the animal were recorded by a revolving kymograph. All experiments were made after a fast of 18 or 21 hours.[1] A series of four to six satisfactory basal tests was always obtained before giving the injection. The injection of the amino acid and the collection of the expired air were started simultaneously. Following this the expired air was collected for 10 minute periods, with from 3 to 5 minute intervals between the tests, over a period of several hours.

The animals were always catheterized in the morning before the basal respiratory tests were begun. At the end of 2 hours, just before the amino acid was given, the bladder was again emptied and washed with physiologic solution of sodium chloride or distilled water. Following the injection, the urine was collected for 2 hour periods and analyzed for total nitrogen, for amino acid nitrogen, and for urea and ammonia nitrogen by the methods of Folin.

Results.

Experiment 1. Alanine.—Fig. 1 shows the results obtained in a typical experiment with alanine. Six basal tests give an average of 24.8 calories each hour, with a maximum of 25.6 calories and a minimum of 23.9. Following the basal period, 10.235 gm. of alanine dissolved in 70 cc. of physiologic sodium chloride solution (0.1 gm. of amino acid nitrogen for each kilo) were injected at the rate of 7 cc. each minute. Following the injection, tests were run in rapid succession for 3 hours and 54 minutes, with a maximal

[1] All of the animals employed in metabolism work are kept on the standard diet described by Lusk which consists of 44 per cent ground beef heart, 44 per cent cracker meal, 8 per cent lard, and 4 per cent bone ash. The basal requirement plus 50 per cent for maintenance was found to be ample to maintain the animals in constant weight.

interval of 5 minutes between tests. At the end of this time a catheterized specimen of urine was obtained, the mask removed, and the animal allowed to walk quietly about the small gasometer room for about half an hour. The mask was then readjusted and a 22 minute rest period given before the next test was started. Eight more tests were then made, followed by catheterization,

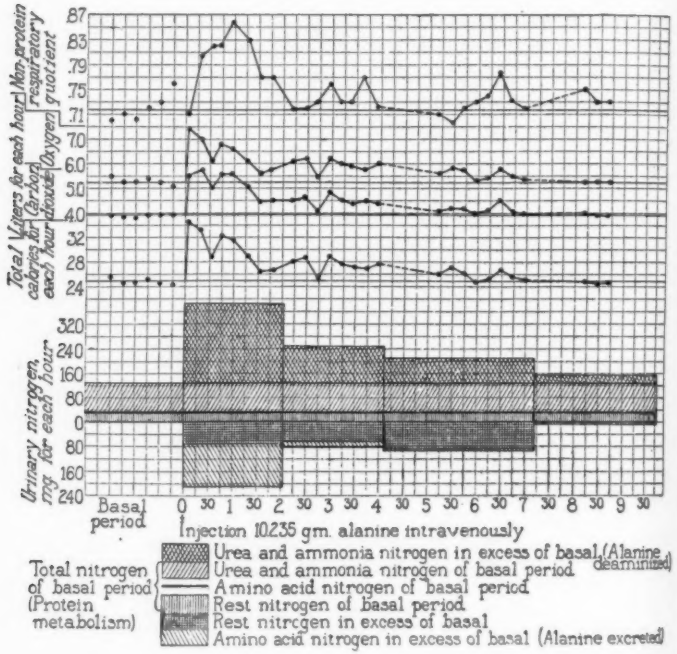

FIG. 1.

another half hour period away from the table, and a 23 minute preliminary rest period before the tests were again started. The total time of the experiment was 8.72 hours.

The test obtained during injection rises sharply from about 25 to 34.8 calories each hour and represents the highest value obtained. Following this the general trend of the curve is downward, until finally the basal value is again reached 8 hours and

8 minutes after the injection. Oxygen consumption and carbon dioxide elimination closely parallel the curve for total calories.

In the calculation of the non-protein respiratory quotient, it was assumed that the total urinary nitrogen of the basal period remained constant throughout the experiment and that it represented the protein metabolism. The factors employed for alanine are as follows:

Physiologic value of 1 gm. of alanine when burned in the body = 3.549 calories.

Calorific value of 1 liter of oxygen used in combustion of alanine = 4.71 calories.

1 gm.	alanine	nitrogen	in	urine	=	4.8 liters oxygen.
1 "	"	"	"	"	=	3.99 " carbon dioxide.
1 "	"	"	"	"	=	6.36 gm. alanine.
1 "	"	"	"	"	=	22.57 calories derived from alanine.

These values are practically identical with those given by Lusk (11, 12).

The average non-protein respiratory quotient for the basal period was 0.717 with a maximum of 0.763 and a minimum of 0.695.[2] During injection there was a slight drop in the respiratory quotient to 0.70 followed by a rapid rise to 0.86, 53 minutes after the beginning of the injection. The respiratory quotient then gradually fell and finally reached the basal value 2 hours and 9 minutes after injection. During the remainder of the test the respiratory quotient fluctuated somewhat but was on the average slightly above the basal value. It is important to note that while the maximal height of heat production occurred during injection, the maximal height of the respiratory quotient occurred 53 minutes later.

In Fig. 1 the urinary data have been plotted as mg. of nitrogen eliminated each hour. One specimen of urine was obtained during the basal period and four were obtained after injection. The total nitrogen of the basal period is considered as representing the animal's basal protein metabolism and is assumed to remain

[2] In our routine work we allow only a 15 or 20 minute preliminary rest period after adjusting the mask. This is sufficient in most cases, since the oxygen consumption in the first test is usually only very slightly above the basal value. The respiratory quotient after this interval, however, is usually somewhat low.

constant during the entire period of observation. The increase in the urea plus the ammonia nitrogen in excess of the basal has been considered as representing the amount of alanine deaminized, while the increase in amino acid nitrogen in excess of the basal has been considered as representing the amount of alanine excreted unchanged. In Fig. 1 the total nitrogen of the basal period (protein metabolism) and the increase of the urea plus ammonia nitrogen in excess of the basal level (alanine deaminized) have been plotted above the zero line, since they have been utilized in calculating the non-protein respiratory quotient. The rest nitrogen and the excess amino acid nitrogen have been plotted below the zero line and have not been used in the calculation of the non-protein respiratory quotient. The increase in rest nitrogen following the injection is not easy to explain, but since it closely parallels the urinary volume it may be due partly to a washing out process attending diuresis and possibly in part to accumulated errors in the several determinations.

The hourly rate of excretion multiplied by the number of hours over which the urine specimen was collected gives the total amount of any of the urinary constituents. Calculated in this manner it is found that the sum of the urea plus ammonia nitrogen in excess of the basal for the total period, plus the amino acid nitrogen in excess of the basal for the total period, amounts to 1.46 gm. of nitrogen. The alanine injected (10.235 gm.) contained 1.61 gm. of nitrogen; the recovery of 1.46 gm. therefore represents approximately 91 per cent for a period of 9 hours and 48 minutes. Of the total amount of alanine accounted for, 77 per cent was deaminized, that is, accounted for as urea plus ammonia nitrogen, while 23 per cent was apparently excreted unchanged. We are well aware that this method of calculation is open to several objections, based primarily on the lag in excretion by the kidneys; nevertheless, the results seemed accurate enough to justify further calculation.

In the determination of the specific dynamic action following injection, a method developed by Ito in the metabolism laboratory of the Mayo Clinic was used (3). It consists in the determination, by means of a previously standardized planimeter, of the area lying between the curve for total calories after injection and the line corresponding to the basal value. In this experiment the

TABLE I.

Injection of 5.56 Gm. of Glycocoll.

Dog 2, weight 10.4 kilos; September 20, 1927.

Time, actual.	Total O_2 per hr.	Non-protein R.Q.	Total calories per hr.	Ventilation per min.	Pulse.	Respiration.	Time after start of injection.
a.m.	*liters*			*liters*			
9.15– 9.25	4.794	0.799	22.9	2.75	97	15	
9.30– 9.40	4.626	0.810	22.2	2.83	89	15	
9.45–10.00	4.350	0.810	20.9	2.89	87	17	
10.05–10.15	4.422	0.821	21.2	3.44	82	19	
10.32	Catheterized urine, Specimen 1.						
10.32–10.42	4.872	0.882	23.6	2.98	91	16	During injection.
10.45–10.55	5.364	0.847	25.9	3.88	77	15	18 min.
10.59–11.09	5.700	0.859	27.6	4.87	71	31	32 "
11.12–11.22	5.766	0.937	28.4	5.06	75	27	45 "
11.26–11.36	5.802	0.938	28.6	4.81	85	29	59 "
11.40–11.50	5.280	0.893	25.7	3.96	81	23	1 hr., 13 min.
11.55 a.m.–12.05 p.m.	5.256	0.836	25.3	4.07	83	26	1 " 28 "
p.m.							
12.10–12.25	5.520	0.779	26.1	3.67	80	21	1 " 45 "
12.35	Catheterized urine, Specimen 2.						
12.36–12.46	5.568	0.708	26.1	3.17	84	16	2 hrs. 9 min.
12.50– 1.00	5.748	0.709	26.9	3.32	85	20	2 " 23 "
1.05– 1.15	5.256	0.695	24.6	3.15	78	20	2 " 38 "
1.20– 1.30	5.574	0.720	26.2	3.84	76	20	2 " 53 "
1.35– 1.45	4.898	0.762	23.2	3.38	73	18	3 " 8 "
1.50– 2.00	5.130	0.695	24.0	3.17	71	17	3 " 23 "
2.20– 2.30	4.860	0.752	22.9	3.19	67	20	3 " 53 "
2.38	Catheterized urine, Specimen 3.						
2.41– 2.51	4.824	0.770	22.8	3.18	73	19	4 hrs. 14 min.
2.55– 3.10	4.386	0.730	20.6	2.82	73	17	4 " 30 "
3.15– 3.30	4.272	0.730	20.0	2.60	67	17	4 " 50 "
3.35– 3.50	4.572	0.720	21.4	2.63	71	15	5 " 10 "
3.55– 4.10	4.452	0.743	20.9	2.59	74	15	5 " 30 "
4.15– 4.30	4.644	0.684	21.7	2.43	74	12	5 " 50 "
4.38	Catheterized urine, Specimen 4.						
4.40– 4.55	4.452	0.802	21.2	2.50	80	13	6 hrs. 15 min.
5.00– 5.15	4.596	0.745	21.6	2.39	79	12	6 " 35 "
5.20– 5.30	4.416	0.744	20.7	2.34	75	11	6 " 53 "
5.45	Catheterized urine, Specimen 5.						

TABLE II.

Injection of 5.56 Gm. of Glycocoll.

Dog 2; September 20, 1927.

Urine specimen No.	Time.	Nitrogen partition products of urine.			Rate of excretion per hr.			Glycocoll deaminized (urea + ammonia N in excess of basal).	Glycocoll excreted (amino acid N in excess of basal).
		Total N.	Urea + ammonia N.	Amino acid N.	Total N.	Urea + ammonia N.	Amino acid N.		
		mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
1	Basal, 8.15–10.32 a.m. (2.28 hrs.)	180	135	2.9	79	59	1.3		
2	After injection, 10.32 a.m.–12.35 p.m. (2.05 hrs.)	418	304	57.0	204	148	27.8	$89 \times 2.05 = 182$	$26.5 \times 2.05 = 54.3$
3	After injection, 12.35–2.38 p.m. (2.05 hrs.)	352	333	5.9	172	163	2.9	$104 \times 2.05 = 213$	$1.6 \times 2.05 = 3.3$
4	After injection, 2.38–4.38 p.m. (2.00 hrs.)	429	403	1.7	215	202	0.9	$143 \times 2.00 = 286$	
5	After injection, 4.38–5.45 p.m. (1.12 hrs.)	150	138	1.4	134	123	1.3	$64 \times 1.12 = 72$ Total $= 753$ 58 811^*	57.6

*0.811 gm. of glycocoll nitrogen recovered which equals 78 per cent of 1.04 gm. given. 93 per cent of the glycocoll accounted for was deaminized and 7 per cent excreted.

specific dynamic action amounts to 21.38 calories for a period of 8 hours and 43 minutes. During this same period the alanine deaminized could have yielded 24.67 calories. The specific dynamic action, therefore, is 87 per cent of the heat value of the alanine deaminized.

Experiment 2. Glycocoll.—Tables I and II give the results of a typical experiment with glycocoll. The dog used in this experiment weighed 10.4 kilos. Four satisfactory basal tests average 21.8 calories and vary between 20.9 and 22.9 calories each hour. 5.56 gm. of glycocoll (0.1 gm. of nitrogen for each kilo) dissolved in 50 cc. of water were injected intravenously in 9 minutes. The test obtained during injection rises from the average basal value of 21.8 to 23.6 calories each hour. Following this there is a progressive rise to 28.6 calories each hour which is reached 59 minutes after the beginning of the injection. After this peak, the curve for total calories forms a broad plateau with a gentle slope downward and finally returns to the basal value 4 hours and 20 minutes after the beginning of the injection. The experiment was continued for slightly more than 2 hours after the basal value had been reached and the last eight tests varied between 20 and 21.7 calories with an average of 21.1 calories, which is practically identical with the average basal value of 21.8 calories before injection.

In the calculation of the non-protein respiratory quotient the following values for glycocoll, which are the same as those given by Lusk (11, 12), were used:

Physiologic value of 1 gm. of glycocoll in body = 2.099 calories.
Calorific value of 1 liter of oxygen used in combustion of glycocoll = 4.685 calories.

1 gm. glycocol	nitrogen	in	urine	=	2.4	liters oxygen.
1 " "	"	"	"	=	2.4	" carbon dioxide.
1 " "	"	"	"	=	5.35	gm. glycocoll.
1 " "	"	"	"	=	11.24	calories derived from glycocoll.

The non-protein respiratory quotient rises from an average basal value of 0.81 to 0.94, 45 minutes after injection and then falls below the basal value for the remainder of the experiment. The maximal height of the respiratory quotient in this experiment coincides in time with the maximal height of extra heat production.

One specimen of urine was obtained during the basal period and four specimens of urine were obtained after injection. When

calculated as already described, it is found that the increase in urea plus ammonia nitrogen over the basal value plus the increase in amino acid nitrogen in excess of the basal, for a period of 7 hours and 13 minutes, gives a recovery of 0.811 gm. of nitrogen which is equivalent to 78 per cent of the nitrogen injected (1.04 gm.). Of the nitrogen thus accounted for, 93 per cent appears as excess urea nitrogen (glycocoll deaminized) while 7 per cent appears as excess amino acid nitrogen (glycocoll excreted).

The curve for total calories returns to the basal value 4 hours and 20 minutes after injection and the specific dynamic action for this period amounts to 15.2 calories. During this same period 2.31 gm. of glycocoll were deaminized and could have yielded 4.8 calories; the specific dynamic action amounts to slightly more than 3 times this figure. The total amount of glycocoll given (5.56 gm.) could have yielded 11.7 calories, and the specific dynamic action amounts to nearly 1½ times this value.

Experiment 3. Phenylalanine.—Because of its relative insolubility phenylalanine presented some difficulty. It was injected after having been dissolved in sodium hydroxide, weak hydrochloric acid, and finally in a large volume of distilled water.

Tables III and IV show the results of an experiment in which 4 gm. of phenylalanine, dissolved in 250 cc. of water, were given intravenously to a dog weighing 5.7 kilos. This was equivalent to about 0.06 gm. of nitrogen for each kilo of body weight. The injection was given slowly and required 1 hour and 11 minutes for completion. Heat production showed a prompt increase and reached a maximum just before the end of the injection. This peak may be somewhat higher than the true value because of slight nausea. All other tests, however, were satisfactory. Heat production was still elevated above the basal level at the end of 4 hours.

The observed respiratory quotient is at first lowered, a phenomenon which is also seen in the experiment with alanine, but which is possibly brought out more clearly in this instance by the slow rate of injection. The initial fall in quotient is not accompanied by a decrease in total ventilation (Table III). Following this the quotient rises to a maximal height of 0.84 and remains elevated above the basal value for 1 hour. The observed respiratory quotient with its corresponding secondary calculations is

given instead of the non-protein, because of the uncertainty in factors for the metabolism of phenylalanine. The total specific

TABLE III.

Injection of 4 Gm. of Phenylalanine in 250 Cc. of Water.

Dog 3, weight 5.7 kilos; April 22, 1927.

Time, actual.	Total O_2 per hr.	Observed R.Q.	Total calories per hr.	Ventilation per min.	Pulse.	Respiration.	Time after start of injection.
a.m.	*liters*			*liters*			
9.20– 9.30	2.808	0.75	13.3	0.113	79	13	
9.35– 9.45	2.670	0.75	12.7	0.111	70	15	
9.50–10.00	2.748	0.75	13.0	0.115	67	13	
10.05–10.15	2.376	0.77	11.3	0.100	67	12	
10.32	Catheterized urine, Specimen 1.						
10.32–10.42	2.952	0.73	13.7	0.121	75	17	During injection.
10.46–10.56	2.868	0.75	13.6	0.125	72	23	" "
10.59–11.10	3.006	0.74	14.2	0.132	70	20	" "
11.15–11.25	3.354	0.78	16.0	0.153	73	18	" "
11.30–11.40	3.912	0.84	19.0	0.203	72	21	" "
11.45–11.55	3.252	0.81	15.7	0.185	72	26	Injection ended 11.42 a.m.
p.m.							
12.00–12.10	2.928	0.84	14.2	0.170	72	22	1 hr. 28 min.
12.15–12.25	3.042	0.83	14.7	0.169	78	19	1 " 43 "
12.35	Catheterized urine, Specimen 2.						
12.37–12.47	2.868	0.76	13.6	0.139	70	15	2 hrs. 5 min.
12.52– 1.02	2.904	0.77	13.8	0.135	69	15	2 " 20 "
1.07– 1.17	3.144	0.75	14.9	0.143	68	14	2 " 35 "
1.22– 1.32	3.078	0.77	14.7	0.142	69	14	2 " 50 "
1.37– 1.47	3.270	0.75	15.5	0.148	71	15	3 " 5 "
1.52– 2.02	3.156	0.75	14.9	0.142	68	15	3 " 20 "
2.07– 2.17	2.964	0.75	14.0	0.135	69	14	3 " 35 "
2.22– 2.32	2.898	0.74	13.7	0.131	69	14	3 " 50 "
2.37– 2.47	2.838	0.74	13.4	0.127	70	13	4 " 5 "
3.00	Catheterized urine, Specimen 3.						

dynamic action in this experiment amounts to 9.3 calories for a period of 4 hours and 5 minutes.

TABLE IV.

Injection of 4 Gm. of Phenylalanine in 250 Cc. of Water.

Dog 3; April 22, 1927.

Urine specimen No.	Time.	Nitrogen partition products of urine.			Rate of excretion per hr.			Phenylalanine deaminized (urea + ammonia N in excess of basal).	Phenylalanine excreted (amino acid N in excess of basal).
		Total N.	Urea + ammonia N.	Amino acid N.	Total N.	Urea + ammonia N.	Amino acid N.		
		mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
1	Basal, 8.30–10.32 a.m. (2.03 hrs.)	152.5	107.5	0.75	75.4	53.2	0.37		
2	After injection, 10.32 a.m.–12.35 p.m. (2.05 hrs.)	257.5	230.0	25.3	126.0	112.0	12.4	$58.8 \times 2.05 = 120.5$	$12 \times 2.05 = 24.6$
3	After injection, 12.35–3.00 p.m. (2.4 hrs.)	192.0	180.0	8.4	80.2	75.0	3.6	$21.8 \times 2.40 = 52.3$	$3 \times 2.40 = 7.2$
								Total $= 172.8$	31.8
								31.8	
								204.6*	

*0.205 gm. of phenylalanine nitrogen recovered which equals 60.3 per cent of the 0.340 gm. given. 84 per cent of the amount of phenylalanine accounted for was deaminized and 16 per cent excreted.

One specimen of urine was obtained during the basal period and two specimens of urine were obtained after injection. Calculating as before, we find that 60 per cent of the nitrogen injected is accounted for by the increase in the urea plus ammonia and the amino acid nitrogen above the basal level. 84 per cent of the phenylalanine thus accounted for was deaminized and 16 per cent excreted.

In a control experiment 300 cc. of physiologic solution of sodium chloride were injected intravenously into a dog weighing 9.1 kilos, at the rate of 3.5 cc. each minute, and an increase in heat production was found which amounted to about 2 calories each hour over a period of 1 hour. This small increase in heat production was primarily the result of restlessness caused by a distended bladder since no urine was collected in this control experiment. The respiratory quotient showed no change.

Other experiments with phenylalanine dissolved in sodium hydroxide gave similar results and will not be given in detail at this time.

DISCUSSION.

The immediate rise in heat production which follows the intravenous administration of amino acid has been observed by Aub, Everett, and Fine (2) and by Wolf and Hele (17) in decerebrate animals, and by Krzywanek in normal animals (8). In Krzywanek's experiments the injections were given very rapidly and the increase in metabolism was noted from 5 to 7 minutes later. Weiss and Rapport (15) concluded that the specific dynamic action of glycine is essentially the same whether it is administered by mouth, subcutaneously, or intravenously. Liebeschütz-Plaut and Schadow (10) failed to obtain evidence of specific dynamic action when glycine and alanine were given intravenously, but did obtain it when they were given by duodenal infusion. Their experiments seem to have been well controlled and it is difficult to explain the negative results obtained. The values for basal heat production, however, seem somewhat high and this may explain their results, since we have frequently noticed that when an animal is, for any reason, considerably above its standard level of heat production, the specific dynamic action is likely to be less or, more strictly speaking, masked.

XUM

The rise of the respiratory quotient after the injection of an amino acid is interesting, but the mechanism of its production is not clear. The occurrence is not peculiar to intravenous administration; it may also follow oral administration as shown by the experiments of Lusk (11, 12) and Krzywanek (8). Following oral administration the respiratory quotient may rise to, or above, the theoretic respiratory quotient of the amino acid, and Williams, Riche, and Lusk (16) have called attention to the fact that after the feeding of meat the respiratory quotient may be higher than that for protein. There are several explanations: (1) It may be due to a disturbance of acid-base equilibrium in the body; this is the explanation given by Weiss and Rapport who found a quotient of 1.25 half an hour after the intravenous injection of glycocoll; (2) it may be due to the combustion of glucose derived from the amino acid *per se;* against this, however, is the result which we have obtained in one experiment with phenylalanine in which a definite rise in respiratory quotient occurred; this amino acid, according to Dakin (5), is not converted to glucose in the phlorhizinized animal; further studies are now in progress to ascertain whether or not a rise in respiratory quotient always follows the administration of phenylalanine; and (3) it may represent a true stimulation of carbohydrate metabolism brought about by the amino acid. Aub, Everett, and Fine (2) emphasize that the rise in quotient may occur without a corresponding rise in heat production, for example, in urethanized cats. We have also observed this dissociation of the two phenomena under several conditions.

The manner in which the specific dynamic action is calculated and expressed is of great importance, especially when secondary calculations are to be made from the data. When repeated single determinations of heat production are made it is a common error to evaluate only the highest point of the curve for total calories and to consider it as representing the specific dynamic action, which is then expressed as the percentage increase of this peak above the basal level. Results expressed in this manner lose much of their value because it is quite obvious that if detailed secondary calculations are to be made, it is absolutely essential to know the total increase in calories over a given period of time. By means of the method developed by Ito, which has already

been mentioned, the total specific dynamic action in calories can be calculated from the curves as illustrated in Fig. 1. When the total calories of specific dynamic action are known, the result is then often expressed as the percentage increase of the extra calories over the total number of calories which would have been produced in the same period of time at a constant basal level of heat production. This method of expression is open to criticism from two different view-points: (1) The result obtained is obviously altered by the length of the experiment, since the curve for extra calories rises to a maximum shortly after the administration of the amino acid and then returns slowly to the basal value as the effect subsides; during this same period of time the basal level of heat production is considered as remaining constant; the ratio between the total extra calories and the total basal calories will therefore depend on how much of the curve has been included in the experimental period, and (2) this method of expression makes it difficult to compare the results obtained with the same amino acid in different animals, since the same percentage increase above the basal value represents an entirely different absolute value for total extra calories produced, depending on the value of the basal heat production. It therefore seems advisable to attempt to correlate the specific dynamic action with the amino acid producing it.

In expressing the relationship between the specific dynamic action in calories and the amount of amino acid which participated in its production, two general courses are open: (1) the consideration of the total amount of amino acid given, and (2) the amount deaminized. Both methods of comparison are open to certain criticisms, since they represent the two extremes. In general, however, the amount deaminized will probably more nearly approximate the true relations in spite of the fact that the rate of elimination of urea over short periods does not exactly represent the amount of amino acid deaminized in such periods. The relationship between the amino acid and the resulting specific dynamic action can be expressed in six different ways: (1) as calories for each millimol of amino acid given, (2) as calories for each millimol deaminized, (3) as calories for each gm. of the amino acid given, (4) as calories for each gm. deaminized, (5) as per cent of the physiologically available calories in the total quan-

tity given, and (6) as per cent of the physiologically available calories in the total quantity deaminized.

These six methods of comparison have been tabulated in Tables V and VI which include, besides the three experiments reported in

TABLE V.

Comparison of Specific Dynamic Action of Various Amino Acids.

Experiment No.	Substance.	Specific dynamic action in calories for:				Specific dynamic action in per cent of:		Per cent of amount given deaminized during time of respiratory experiment.	Length of experiment.	Remarks.
		Each mM given.	Each mM deaminized.	Each gm. given.	Each gm. deaminized.	Calories given.	Calories in amount deaminized.			
									hrs.	
3	Phenylalanine.	0.39	0.78	2.3	4.8	37	76	48	4.08	4 gm. in 250 cc. H_2O.
4	"	0.31	0.62	1.8	3.7	29	59	49	3.41	10 gm. in 200 cc. H_2O and 5 cc. saturated NaOH.
2 A	Glycocoll.	0.20	0.49	2.7	6.5	130	316	42	4.33	5.56 gm. in 50 cc. H_2O.
5	"	0.12	0.41	1.7	5.6	80	269	30	3.95	6.20 gm. in 50 cc. H_2O.
6	Alanine.	0.12	0.31	1.3	3.5	36	98	37	4.18	10.43 gm. in 100 cc. physiologic solution of NaCl.
1 A	"	0.13	0.39	1.5	4.3	42	121	35	4.00	10.235 gm. in 100 cc. physiologic solution of NaCl.
1	"	0.19	0.28	2.1	3.1	59	87	68	8.72	" "
2	Glycocoll.	0.20	0.29	2.7	3.9	130	185	70	6.88	5.56 gm. in 50 cc. H_2O.

detail in this paper, one additional experiment with each amino acid. In Experiment 4 with phenylalanine, sodium hydroxide was employed as a solvent and caused marked initial lowering of the respiratory quotient, followed later by a rise which was less

TABLE VI.

Summary of Data Used in Making Calculations in Table V.

Experiment No.	Substance.	Specific dynamic action in calories.	mM given.	mM deaminized.	Gm. given.	Gm. deaminized.	Calories in amount given.	Calories in amount deaminized.	Molecular weight.	Calorific value, 1 gm. in calorimeter.	Calorific value of urea from 1 gm.	Physiologic value for:	
												1 gm.	1 mM.
3	Phenylalanine.	9.3	24	11.6	4	1.93	25.2	12.2	165.15	6.752*	0.457*	6.295*	1.05
4	"	18.4	60	29.6	10	4.94	63.0	31.1	165.15	6.752*	0.457*	6.295*	1.05
2 A	Glycocoll.	15.2	74	31	5.56	2.31	11.7	4.8	75.06	3.110†	1.011	2.099	0.16
5	"	10.37	83	25	6.20	1.84	13.01	3.86	75.06	3.110†	1.011	2.099	0.16
6	Alanine.	13.46	117	43	10.43	3.87	37.02	13.7	89.1	4.401†	0.852	3.549	0.32
1A	"	15.27	114	39.6	10.235	3.55	36.32	12.6	89.1	4.401†	0.852	3.549	0.32
1	"	21.38	114	77.4	10.234	6.95	36.32	24.66	89.1	4.401†	0.852	3.549	0.32
2	Glycocoll.	15.2	74	52.0	5.56	3.91	11.7	8.21	75.06	3.110†	1.011	2.099	0.16

* Rapport and Beard (14).
† Emery and Benedict (6).

marked than in Experiment 3 in which the solvent was distilled water. Because of this, the calories calculated from the respiratory quotient are somewhat lower in Experiment 4. The experiments with alanine (Experiment 1) and with glycocoll (Experiment 2), which are reported in detail in this paper, lasted for 8.72 hours and 6.88 hours respectively, and in Table V the data from these experiments have been calculated both for the total period of time and also for 4 hours (Experiment 1A) and for 4.33 hours (Experiment 2A); the latter values are then quite comparable, on the basis of time, with the other experiments summarized in Table V which lasted for periods of time ranging from 3.41 hours to 4.18 hours.

Of the six methods for comparing specific dynamic actions which have been enumerated above, the two most commonly employed are as calories for each gm. of amino acid given or for each gm. deaminized. As calories for each gm. given it is seen that there is no essential difference between glycocoll and phenylalanine; the average value for alanine, however, is somewhat below the average value for glycocoll and phenylalanine, although the high value for alanine (1.5 calories) is of the same order of magnitude as the low values of 1.7 and 1.8 calories for glycocoll and phenylalanine. As calories for each gm. deaminized the values for alanine and phenylalanine are practically identical while the values for glycocoll are somewhat greater. If these values are compared with the values expressed as calories for each millimol given or each millimol deaminized, a striking difference is noted. In spite of certain criticisms, which have been mentioned, it seems likely that the relationship is more correctly expressed as calories of specific dynamic action for each millimol deaminized. The amino acids are undoubtedly utilized by the body as individual molecules, not as gm. or calories, and a method of comparison which takes this fact into account has much in its favor. When the values are expressed as calories for each millimol deaminized it is seen that these three amino acids become clearly grouped, alanine having the least powerful and phenylalanine the most powerful influence on metabolism. The difference between alanine and glycocoll is not marked, the average value for alanine being 0.35 calories for each millimol and that for glycocoll 0.45 calories. The average value for phenylalanine, however, is approximately 1.6 times the

average for glycocoll and twice that of alanine. When the differences in the molecular weights are considered, it becomes evident why the value for phenylalanine, which is twice that of alanine when expressed as calories for each millimol deaminized, becomes practically the same when expressed as calories for each gm. deaminized. The molecular weight of phenylalanine is approximately twice that of alanine; 1 gm. therefore contains approximately half as many molecules, but since each molecule exerts twice the effect on metabolism, the values will be approximately equal when expressed as calories for each gm. The same method of reasoning explains why glycocoll is apparently more potent than phenylalanine when they are compared as calories for each gm. deaminized.

In the seventh and eighth columns of Table V the specific dynamic action is expressed as per cent of the physiologically available calories in the total quantity of amino acid given and in the amount deaminized; that is, the calories of specific dynamic action have been divided by the physiologically available calories in the total quantity given or in the quantity deaminized. The "physiologic heat value" of 1 gm. of an amino acid is the caloric value of 1 gm. as determined in the calorimeter, minus the caloric value of the quantity of urea theoretically derived from 1 gm. In the case of glycocoll the heat value of the urea which is subtracted amounts to about $\frac{1}{3}$ of the calorimeter value for 1 gm. of the amino acid, with alanine about $\frac{1}{5}$, while with phenylalanine it is only about $\frac{1}{13}$. This difference in the relative proportion of heat which is subtracted will at least in part explain why the specific dynamic action of these three amino acids, which are in the approximate ratio of 1 to 1.3 to 2 for alanine, glycocoll, and phenylalanine, when compared on the basis of the number of calories of extra heat produced by each millimol of the amino acid deaminized, becomes approximately 1 to 3 to 0.7 for alanine, glycocoll, and phenylalanine when expressed as:

$$\frac{\text{Calories of specific dynamic action}}{\text{Physiologically available calories in amount deaminized}}$$

Still another possible source of error is brought to light when this method of calculating the physiologically available calories is applied to phenylalanine; in this case a certain undetermined

fraction may be excreted as a free or combined phenol and the calorie value of 1 gm. still further reduced. The careful studies of Lusk (13) led him to state that 100 per cent of the energy contained in glycocoll and 50 per cent of that in alanine may appear as extra heat when they are given by mouth. Our values for specific dynamic action expressed in per cent of calories given (Table V) agree fairly well with Lusk's figures, the average value amounting to 105 per cent of the energy in the total quantity of glycocoll given, while alanine yielded 36 and 42 per cent in 4 hours and 59 per cent in 8.72 hours.

When the values are expressed as per cent of calories in amount deaminized it is seen that with glycocoll the extra heat produced is about 3 times the calorie value of the amount of glycocoll deaminized. Since the amount deaminized was calculated from the "extra" urea which was eliminated during the relatively short period of approximately 4 hours, it is possible that the lag in urea excretion may in part account for the high value obtained; however, the amount of glycocoll deaminized during the experiments amounted to 30 and 42 per cent which is of about the same order of magnitude as the value obtained for alanine and phenylalanine. When Experiment 1, with alanine, is calculated for a period of 4 hours (Experiment 1A), it is likewise found that the heat appearing as specific dynamic action is definitely in excess (121 per cent) of the heat value of the amount of alanine deaminized.

Rapport and Beard (14) apparently first called attention to the high value of the specific dynamic action of phenylalanine. We have recalculated two of their experiments and obtain values of 0.32 and 0.35 calories of specific dynamic action for each millimol given and 0.62 and 0.54 calories for each millimol deaminized as compared with 0.31 and 0.39 calories for each millimol given and 0.62 and 0.78 calories for each millimol deaminized in our experiments. These results are all in fair agreement when the differences in technique and in the mode of administration of the amino acids are considered. The fact that the specific dynamic action of phenylalanine for each millimol deaminized is approximately twice as great as that of alanine, permits a suggestion regarding the part played by the two portions of the phenylalanine molecule in producing the increase in heat production, for if we assume that the alanine fraction will have the same effect as alanine when

administered alone, then it follows that the phenol fraction of the molecule must exert an equally great effect on heat production.

In our experiments with alanine and phenylalanine we used the racemic forms and it is important to note that apparently a portion of both *d* and *l* components was deaminized. In Experiment 1, with alanine, the urine was collected for 9.79 hours after the injection and during this time 1.124 gm. of extra urea nitrogen were eliminated. The quantity of alanine injected contained 1.61 gm. of nitrogen and we may consider 50 per cent of this (0.805 gm.) as the nitrogen of *d*-alanine and a like quantity as nitrogen of *l*-alanine. If it is assumed that all of one component was deaminized, then it follows that approximately 40 per cent of the other component was likewise deaminized. Levene and Meyer (9) fed racemic alanine, along with a standard diet, to a dog, and concluded that all of the *l*-alanine and 68 per cent of the *d*-alanine was deaminized. Abderhalden and Schittenhelm (1) performed similar feeding experiments with racemic alanine and from their data one may likewise calculate that all of one component and about 69 per cent of the other was deaminized. In our Experiment 3 with phenylalanine (Table IV) a similar calculation permits the conclusion that all of one component plus 21 per cent of the optical antipode was deaminized. Levene and Meyer obtained comparable results with phenylalanine. These considerations bring up an interesting question which unfortunately cannot be answered at the present time, namely: Are the specific dynamic actions of the *d* and *l* forms of an amino acid the same? If the values differ, then experiments performed with the racemic forms, which obviously give only the sum of the two effects, may be expected to differ at times, depending on the relative quantities of the two components which participate in the reaction.

In conclusion we wish to emphasize two points with reference to the interpretation of our results: (1) We do not believe, nor do we wish to imply that specific dynamic action is necessarily always associated with deaminization of the amino acid, and (2) the values for specific dynamic action given in this paper are applicable only to normal animals on a standard, fully adequate diet and with the same rates of injection which we have employed in these experiments.

SUMMARY.

Following the intravenous injection of alanine, glycocoll, and phenylalanine, there is an immediate rise in heat production which usually reaches its height during the injection and with glycocoll and alanine may require from 4½ to 9 hours to return to the basal value.

Accompanying the increased heat production there is a definite elevation of the respiratory quotient.

The relationship between the specific dynamic action and the amino acid administered may be expressed in at least six different ways and reasons are given for believing that the most suitable manner of expressing this relationship is as calories of extra heat produced by each millimol of the amino acid deaminized. Expressed in this manner the specific dynamic action of alanine, glycocoll, and phenylalanine are in the approximate ratio of 1 to 1.3 to 2.

When racemic forms of amino acids are injected, more than 50 per cent of the nitrogen may appear in the urine as "extra" urea nitrogen, thus indicating that all of one component plus a certain varying fraction of the optical antipode has been deaminized.

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THE EFFECT OF INSULIN ON THE AMINO ACID CONTENT OF BLOOD.

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INTRODUCTION.

Early in 1926, one of us (J. M. L.) had occasion to determine the amino nitrogen content of a number of blood samples from fasting pathological subjects. Among them were some diabetic cases under treatment with insulin. With only four or five exceptions, this latter group gave the lowest amino nitrogen values observed. Although there were factors other than insulin which may have contributed to the difference in group values between diabetics and non-diabetics, the results were sufficient to excite our curiosity, and to lead us to an examination of the effect of insulin upon the amino acid content of the blood.

An examination of the literature revealed several points to which reference should be made. In the first place, we could find little agreement in the conclusions drawn by various authors with respect to the amino acid content of the blood of fasting diabetic subjects.

Desqueyroux (1) reported the amino nitrogen content to be greater than normal. Wolpe (2) also found that most of the diabetics examined by him gave high values. Wiechmann's (3) subjects appear to have had no abnormal concentration of amino acids in the blood prior to insulin treatment, although he gave no analyses of normal blood which might have served us as a basis for comparison. The extensive investigations of Greene, Sandiford, and Ross (4) demonstrated that in 116 diabetic subjects the amino nitrogen content of the blood did not depart from normal limits. The results obtained by von Falkenhausen (5) on the blood of diabetic subjects confirm those of Greene, Sandiford, and Ross. Finally there are the investigations of Okada and Hayashi (6) on the blood of depancreatized dogs, and of quite a number of investigators (7) on the amino acid excretion of diabetic subjects which indicated hyperaminoacidemia.

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As for the effects of insulin, the reports were meager and somewhat conflicting. Wolpe (2) appears to have examined the effect of insulin on the blood amino nitrogen of only one diabetic subject. He concluded, however, as did Wiechmann (3) that insulin reduced the amino acid content of the blood of diabetics. The latter's findings were somewhat more extensive than Wolpe's and possess an added interest in that no parallel decreases were observed in the blood sugar. Wiechmann also found that insulin decreased the amino acid content of the urine. Greene, Sandiford, and Ross (4) concluded, without presentation of experimental data, that insulin had no effect on the amino acids (of the blood). Tashiro (8), however, reported the interesting, if not surprising, observation that insulin greatly reduced the amino nitrogen of the blood, even to zero in some cases. His work seems to have been limited to experimentation upon two rabbits, in one of which, and for other purposes, the cervical sympathetic nerves had been cut. Moreover, the values described as pertaining to amino nitrogen referred in reality to the residual nitrogen fraction (non-protein, non-urea).

EXPERIMENTAL.

The effect of insulin upon the amino nitrogen content of the blood of rabbits, rats, and humans was investigated. The rabbits were, in all cases, fasted for 48 hours before use, the rats for 48 hours, and the two human subjects for 16 hours. The insulin employed was Eli Lilly and Company's iletin. In the experiment with rats, the insulin was diluted with 0.9 per cent sodium chloride to such a strength that 1 cc. of the diluted material contained the requisite amount of insulin for injection. Controls received in a similar manner 1 cc. of 0.9 per cent sodium chloride by subcutaneous injection.

The blood of the rabbits was collected from a marginal ear vein, that of the rats by stunning and cutting deeply into the thorax, and that of the humans by puncture of an arm vein. Finely powdered potassium oxalate was employed in all cases as an anticoagulant.

The rats used were raised in our own colony, having been maintained, prior to experimentation, upon the basal diet elsewhere described (9). Only males, weighing 150 to 225 gm. were employed.

The rats were examined in groups of five, all members being simultaneously injected, and then killed in succession at suitable intervals of time.

In the protein-free blood filtrates, prepared according to the

method of Folin and Wu (10), reducing sugar was determined by the method of Benedict (11) and amino acid nitrogen by that of Folin (12). To be quite certain that the changes observed in the

TABLE I.

Effect of Insulin upon Amino Acid Nitrogen in Blood of Man.

Time.	Blood sugar.	Amino N.		Remarks.
		Colorimetric.	Gasometric.	
	mg. per cent	*mg. per cent*	*mg. per cent*	
8.40 a.m.	88	5.6	5.6	L. F. W., 82 kilos.
9.07 "	30 units insulin injected subcutaneously.			
9.37 "	72	5.5	4.7	
10.07 "	59	5.0	4.5	
10.39 "	58	4.9	4.8	
11.26 "	61	5.0	4.0	
12.12 p.m.	59	4.2	3.4	
8.55 a.m.	84	6.4		J. M. L., 69 kilos.
9.22 "	25 units insulin injected subcutaneously.			
9.52 "	58	5.0		
10.24 "	71	4.7		
10.57 "	68	4.8		
11.45 "	61	4.7		

TABLE II.

Effect of Insulin upon Amino Acid Nitrogen in Blood of Rats.

Time.	Amino N, mg. per cent.						Remarks.
	Control series.	Control series.	Series 1.	Series 2.	Series 3.	Series 4.	
hrs.							
0	11.7		10.0	13.6	10.0	12.5	Insulin, 6 units per kilo,
¾	11.2	9.65	11.2	9.9	9.0	10.0	was used in Series 1,
1½		9.5	7.8	9.3	7.6	8.2	3, 4; 9 units per kilo
2¼	12.5	9.7	6.4	8.2	7.8	8.3	in Series 2.
3¼–4½	13.1		9.2	10.3		9.5	

amino acid values were not artifact, due to some curious vagary of the analytical method employed, duplicate analyses were performed on the blood samples of one subject, the absolute gaso-

metric method of Van Slyke being used for this purpose. The preliminary treatment of the samples so examined was essentially that described by one of us in another paper (9). 10 cc. samples of blood were used, being diluted to final volumes of 50

TABLE III.

Effect of Insulin upon Amino Nitrogen Content of Blood of Rabbits.

Animal No.	Amino N, mg. per cent. Time after injection. 0 hr.	1 hr.	2 hrs.	3 hrs.	4 hrs.	5 hrs.	Remarks.
Control 1.	9.5	9.2	9.6	9.3	8.8	9.5	
" 2.	8.9	8.7	8.3	8.5	8.6		
Experimental 3.	10.0	9.1	7.8	7.6	6.9	6.9	4 units insulin.
" 4.	9.2	7.9	7.9	Animal died.			6 " "
" 5.	9.7	7.8	7.4	6.9	Animal died.		6 " "

	0 hr.	1½-2 hrs.	2½-3½ hrs.	4-5 hrs.	6-7 hrs.	9-10 hrs.	
Control 6.	9.9	9.8		9.8	9.7		
Experimental 7.	9.6	8.4	7.8	7.2	6.6		1 unit insulin per kilo: no convulsions.
" 8.	9.5	7.7	6.4			8.3	2 units per kilo; convulsions from 2 to 4 hrs. after injection.
" 9.	10.5	7.0	6.7	6.6	6.5		1 unit per kilo; no convulsions.
" 10.	10.1	8.0	7.6	9.5	9.9		1 unit per kilo; no convulsions.

cc. before filtration. Liquid air treatment, of course, was omitted. The results of these experiments are presented in Tables I, II, and III. In most cases, we have not considered it necessary to reproduce the blood sugar values, the changes in which as a result of insulin injections, are quite well known.

DISCUSSION.

These results demonstrate beyond doubt that insulin reduces the amino acid content of the blood. Although the explanation of this phenomenon will be considered in a subsequent paper, two facts may be referred to now which are pertinent to the question of the relationship of this effect to the accompanying hypoglycemia.

The first question that arises is whether hypoaminoacidemia may not be a necessary sequel to hypoglycemia. It might be argued, *a priori*, that the hypoglycemic state would serve as a stimulus to those processes which lead to sugar formation, and by which presumably the organism might endeavor to maintain a constant blood sugar level. If this be so, we would expect that hypoglycemia would be associated with an increased catabolism of the glucogenetic amino acids and hypoaminoacidemia would ensue.

A number of investigators (13) have reported that the injection of hydrazine sulfate into animals provokes hypoglycemia. Lewis and Izume (14) studied also the effect of hydrazine sulfate upon the amino acid content of blood. It was amply demonstrated by them that during hydrazine hypoglycemia the amino acid content suffers a marked increase. Blatherwick, Sahyun, and Hill (15) have recently made the interesting observation that synthalin, which provokes hypoglycemia and has for this reason been investigated as a therapeutic agent for the treatment of diabetes, increases the hyperaminoacidemia which follows the administration of glycine. These results demonstrate quite convincingly that hypoglycemia, *per se*, is not necessarily atended by hypoaminoacidemia.

It is also to be observed that the lowering in the amino nitrogen content of the blood, here observed, is not secondary to convulsions or coma. For in most of the experiments, including those on the human subjects, no untoward outward symptoms were manifest.

Finally it might be well to point out that the degree of hypoaminoacidemia relative to the hypoglycemia is much greater than a cursory examination of this data would suggest. If the maximum fall in amino acid nitrogen be considered as 2.5 to 3.0 mg.

per cent under conditions in which the blood sugar is reduced by a maximum of 40 to 50 mg. per cent, it follows that the proportional decreases in gm.-molecules of amino acids and sugar would be as (2.5 or 3.0) $\times \frac{180}{14}$ is to 40 or 50. In other words the effect of insulin upon the amino acid content of blood is roughly 80 per cent as great as its effect upon the blood sugar.

SUMMARY.

1. Subconvulsive doses of insulin lowered the amino acid content of the blood of rabbits, rats, and humans.

2. Probably this reduction was not a direct and inevitable result of the accompanying hypoglycemia.

3. The decreases observed in the molecular concentration of the amino acids of the blood were, in some instances, almost as great as the decreases in the concentration of blood sugar similarly expressed.

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STUDIES UPON CALCIFICATION IN VITRO.

III. INORGANIC FACTORS DETERMINING CALCIFICATION.

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The histological studies of Pommer (1) and Schmorl (2) have shown that in rickets calcification of the provisional zone of cartilage and of the osteoid fails to occur. This accounts for the overgrowth and the random development of the cartilage cells, the excessive production of osteoid about the bony trabeculæ and the periosteum, and lastly the formation of the so called rachitic metaphysis. Feeding experiments have done much to clarify our knowledge of the etiology of this disease, both as it occurs in the experimental animal and in children, but have contributed little in explaining the failure of calcification. Various explanations have been offered for this failure; namely, first, that it is due to a lack of bone-forming elements in the diet; second, that it is due to defective absorption of mineral elements from the gastrointestinal tract, particularly calcium and phosphorus; third, that there is a deficiency of these bone-forming elements in the blood serum; fourth, that it is due to an inability on the part of the cartilage and the osteoid to take up calcium and phosphorus from the serum.

Studies by Howland and Kramer (3) have shown that an adequate amount of serum calcium and inorganic phosphorus is necessary for normal ossification. These authors have demonstrated that when both the product of the serum calcium and inorganic phosphorus expressed in mg. per cent is 30 or less, the normal process of calcification fails to occur and rickets develops. When this product, however, is more than 40, either rickets is absent or healing is taking place. The studies of Shipley, later confirmed by Shipley, Kramer, and Howland (4) have further demonstrated the ability of rachitic cartilage to calcify in the presence of a sufficient amount of bone-forming salts. When a section of the upper part of the tibia of a rachitic rat was immersed either in serum or in an inorganic salt solution having the mineral composition of serum, calcification occurred in the provisional zone of cartilage if the concentration of calcium and inorganic phosphorus of the medium was within normal limits. If, however, the

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product of the concentration of these components was less than 35, calcification failed to take place.

The observation of Shipley and his coworkers that calcification in the provisional zone may be brought about in inorganic salt solutions under optimal conditions, and that if fails to occur when these conditions are changed, offers a method by which the factors which underlie calcification may be determined. It would follow that if calcification *in vitro* is similar, if not identical, with that occurring *in vivo*, the factors that operate in the artificial serum may have their analogy in the living organism. It was hoped that any results that might materialize from such a study would shed some light on the mechanism of pathological calcification, the process of normal ossification, and the factors causative of such diseases as rickets and osteomalacia. In its histological appearance and location *in vitro* calcification strongly resembles the primary calcification which develops *in vivo* after the administration of an antirachitic factor.

Hastings, Murray, and Sendroy (5) have derived formulas for calculating the activities of $[Ca^{++}]$, $[PO_4^{\equiv}]$, and $[HCO^{-}]$ from the solubilities of their salts at varying ionic strengths and reactions in aqueous solutions. They have compared the results obtained with inorganic solutions with those found in fresh serum and serum after equilibration with solid $CaCO_3$, $Ca_3(PO_4)_2$, and mixtures of these salts as well as with bone powder. They obtained large discrepancies in the figures for calcium ion activity obtained by these methods. They were unable to demonstrate the high degree of supersaturation of serum reported by Holt, La Mer, and Chown (6). Their values for the true activity solubility product of $CaCO_3$ and $Ca_3(PO_4)_2$ differed considerably from those of Holt and collaborators.

In view of the uncertainty which prevails regarding the quantitative composition of primary calcification, discussions as to the relation between the solubility product constants of certain calcium salts of serum and those of bone must be considered premature.

In the study here reported, the aim has been to determine the factors that govern this process, but instead of using precipitation as the criterion for calcification, the cartilage cell served as an index of the conditions best suited for the deposition of bone salts in its matrix; in this the usage of Shipley, Kramer, and Howland was followed. At least such a method approaches more closely the conditions that may be found in the intact living body. It must be borne in mind, however, that before any conclusions derived from a study of the *in vitro* process can be applied to *in vivo* calcification, it must be shown that the quantitative composition of the material deposited in *in vivo* calcification is identical with that obtained in the *in vitro* process.

Experimental Procedure.

The method employed was essentially that described by Shipley, Kramer, and Howland except that the rats were fed on the Steenbock Diet 2965 (7) instead of on the McCollum Diet 3143 (8).

Solutions of inorganic salts are prepared approximating in composition blood serum or *any variation from it.* 20 cc. of a 0.3 per cent solution of phenolsulfonephthalein per liter are added as an indicator and the reaction adjusted by bubbling CO_2 gas through the solution. The solution is filtered through a Mandler filter into a sterile suction flask and portions transferred to 25 cc. Erlenmeyer flasks and stoppered with rubber stoppers. The reaction of each sample is determined colorimetrically by matching against a series of phosphate standards, and the flasks are then placed in the incubator for 30 minutes.

Young rats from a known breeding stock are kept on the Steenbock Diet 2965 (7) for about 20 days or longer until they show a very wide rachitic metaphysis with not a trace of lime salts in the provisional zone of cartilage nor in the trabeculæ. As soon as the flasks attain a temperature of about 37.5°, a rat is killed by a sharp blow on the head, the whole body is washed with alcohol, and the tibiæ are isolated free of muscle tissue with sterile instruments. A tibia is picked up with a sterile piece of gauze and, surrounded by the gauze, its upper part is held between the left thumb and index finger. With a sharp sterile cataract knife, five or six sections of bone are cut through the epiphysis, metaphysis, and a short distance through the diaphysis. The mouth of the Erlenmeyer flask is flamed by one worker, and the cut section is picked up with a sterile forceps by another worker and placed in the solution. The flask is stoppered and the rubber stopper taped to the neck of the flask with longitudinal and circular strips of adhesive tape. It is then replaced in the incubator until ready for microscopic examination.

At the desired time, usually about 20 hours, the flasks are removed from the incubator, one by one, and the pH and the temperature of the contents noted. The solution is poured off and the section is washed several times in distilled water and then placed in a crystallizing dish containing a 1 per cent solution of $AgNO_3$, and exposed to light. The section is examined under the

binocular microscope and the presence of calcification determined. When calcification occurs, the lime salts deposited in the matrix around the cartilage cells in the provisional zone assume first a yellow, then a brown, and lastly a black color. The appearance of the *in vitro* calcification is that of a honeycomb and is identical with newly calcified cartilage *in vivo*. The narrowing of the zone of cartilage cells with the disappearance of the tongues of cartilage, which occurs in animals treated with antirachitic agents, is of course not present in the *in vitro* process.

It is obvious that with so many kinds of inorganic ions in the solution, it is possible to produce a very large number of solutions of different concentrations. We have not attempted an exhaustive study of the subject but have merely attempted to determine the effect of certain alterations in the concentration of one or more ions or of the total ionic strength of the solution.

A. Effect of Reaction on Calcification.

In a preliminary communication (9) we reported the effect of reaction (pH) on calcification *in vitro*. The solutions employed approximated the inorganic composition of blood serum. The concentrations of all components were kept constant but the pH was varied from the acid to the alkaline side of the physiological range. The results may be summarized as follows: (1) The optimum pH for *in vitro* calcification when the product of the concentrations of calcium and phosphorus expressed in mg. per cent equals 50, is from 7.25 to 7.35. (2) Calcification occurs irregularly in the zone of provisional cartilage at pH 7.1 to 7.2. (3) Slight calcification may occur at pH 7.0 to 7.1. (4) No calcification occurs in solutions more acid than pH 7.0 and the sections appear swollen. (5) In solutions more alkaline than pH 7.35 calcification takes place, provided precipitation does not occur. (6) Sections left in acid solutions for from 2 to 6 hours and later transferred into solutions in which the concentrations of calcium, inorganic phosphorus, and hydrogen ions are optimal, fail to calcify; whereas similar sections transferred from solution of low Ca $\times$ P product into solutions of a high product may calcify if the pH is optimal in both solutions.

B. Effect of Sodium Chloride on Calcification in Vitro.

Chemical studies of the blood in rickets and tetany involved, for the most part, the determination of the calcium and inorganic phosphate of the serum. Very little is known of the effect of other electrolytes of the blood on the activity of the calcium or phosphorus of the serum. The well known effect of increasing concentrations of neutral salts on the solubility of slightly soluble salts and the activity coefficients of their ions, led us to study the effect of such salts on calcification *in vitro*. Solutions were prepared simulating the inorganic composition of blood serum, except that the concentrations of calcium, phosphate, sodium, and chloride ions were varied. To basic solutions containing a given amount of calcium and phosphate, but no NaCl, increasing amounts of NaCl were added so that the concentration of this salt ranged through concentrations less than, equal to, and greater than those which obtain in normal serum.[1] The reaction and the concentrations of all other components were kept constant. The results are shown in Table I. It is seen that in solutions in which reaction is optimal and the concentration of calcium and phosphate adequate ($Ca \times P = 50$) calcification *in vitro* occurred best when the concentration of $[Na^+]$ varied between 50 and 150 mM and that of $[Cl^-]$ between 25 and 125 mM. Calcification is definitely inhibited at high concentration of sodium chloride.

In terms of total molal concentration, it is seen that the optimal conditions for calcification *in vitro* when $Ca \times P = 50$ is between 115 and 315 mM. The process of lime salt deposition is entirely inhibited by higher concentrations of sodium chloride.

The ionic strength, μ, of normal blood serum was estimated by Van Slyke and coworkers (11) to be about 0.167. We have calculated the total ionic strength of our solutions and have found that calcification proceeds regularly when μ is between 0.070 and 0.172. Calcification is partially inhibited when $\mu = 0.195$ and is entirely prevented when $\mu = 0.220$. When the concentrations of calcium and phosphate are diminished, calcification fails to occur even with lower values of μ.

[1] These concentrations of sodium chloride are greater than those found by Shipley and Holt (10) sufficient to inhibit calcification. These investigators, however, worked with lower concentrations of calcium and phosphate.

XUM

TABLE I.

Effect of $[Na^+]$ *and* $[Cl^-]$ *on Calcification in Vitro, at pH 7.25 to 7.35 and 37–38°.* $[K^+]$ *5.7 mM,* $[Mg^{++}]$ *0.75 mM,* $[HCO_3^-]$ *30 mM,* $[SO_4^-]$ *0.75 mM, and Varying* $[Ca^{++}]$ *and* $[PO_4^{=}]$ *Concentrations.*

$[Na^+]$	$[Cl^-]$	$[Ca^{++}]$	$[PO_4^{=}]$	Ca × P	Total mM concentration.	μ*	Calcification.
mM	*mM*	*mM*	*mM*	*mg. per cent*			
34.7	9.0	2.5	1.6	50	85.0	0.0549	++ to +++
50.0	24.3	2.5	1.6	50	115.3	0.0702	1 to 3 (++++)
75.0	49.3	2.5	1.6	50	165.6	0.0952	3 (++++)
100.0	74.3	2.5	1.6	50	215.6	0.1202	3 (++++)
125.0	99.3	2.5	1.6	50	265.6	0.1452	++++
150.0	124.3	2.5	1.6	50	315.6	0.1702	++++
175.0	149.3	2.5	1.6	50	365.6	0.1952	+++
200.0	174.3	2.5	1.6	50	415.6	0.2202	Negative.
34.7	9.0	2.0	1.6	40	84.5	0.0541	Negative.
100.0	73.2	2.0	1.6	40	214.0	0.1189	+ to ++
150.0	123.3	2.0	1.6	40	314.1	0.1689	+++
200.0	173.3	2.0	1.6	40	414.1	0.2189	Negative.
34.7	9.0	2.5	1.3	40	84.7	0.0535	+
100.0	74.2	2.5	1.3	40	215.2	0.1188	++†
150.0	124.3	2.5	1.3	40	315.3	0.1688	++
200.0	174.3	2.5	1.3	40	415.3	0.2188	Negative.
34.7	9.0	2.5	1.1	35	84.5	0.0526	Negative.
100.0	74.2	2.5	1.1	35	215.0	0.1179	"
150.0	124.3	2.5	1.1	35	315.1	0.1679	"
200.0	174.3	2.5	1.1	35	415.1	0.2179	"

+ beginning calcification; ++ more advanced; +++ moderate or nearly complete line of calcification; ++++ denotes calcification completely across the provisional zone of cartilage; 2 and 3 (++++) designate calcification also in primary and secondary tongues of cartilage.

* Total ionic strength. $\mu = \frac{1}{2}(c_1z_1^2+c_2z_2^2+c_3z_3^2+\ldots.c_nz_n^2)$ where $c_1\ c_2\ c_3\ldots.c_n$ = molal concentration of ions present and $z_1\ z_2\ z_3\ldots.z_n$ = valence of ions (11).

†++ in 18 hours, nearly ++++ in 48 hours.

C. Effect of Potassium on Calcification in Vitro.

In the distribution of electrolytes between the erythrocytes and serum, $[K^+]$ is the major cation of the red cell, while $[Na^+]$ is found

exclusively in the serum. The concentration of $[K^+]$ of serum as drawn is about 23 mg. per cent or 5.7 mM, while that of $[Na^+]$ is about 150 mM. If the effect of NaCl in inhibiting calcification were due entirely to an increase in total ionic strength, it would follow that by increasing the total ionic strength by substituting $[K^+]$ for $[Na^+]$ identical results should be obtained. From Table II it is seen that the effect of $[K^+]$ on calcification is parallel with that observed when $[Na^+]$ is employed. This seems to indicate

TABLE II.

Effect of $[K^+]$ on Calcification in Vitro at pH 7.25 to 7.35 and 37–38°. $[Ca^{++}]$ 2.5 mM, $[PO_4^{=}]$ 1.6 mM, $[Mg^{++}]$ 0.75 mM, $[SO_4^{=}]$ 0.75 mM, $[HCO_3^-]$ 30 mM.

$[Na^+]$	$[Cl^-]$	$[K^+]$	Ca × P	Total mM concentration.	μ	Calcification.
mM	*mM*	*mM*	*mg. per cent*			
53.4	60.8	1.7	50	151.5	0.0881	2 (++++)
103.3	116.3	1.7	50	256.9	0.1408	2 (++++)
201.6	230.0	1.7	50	468.9	0.2468	Negative.
50.0	24.3	5.7	50	115.6	0.0702	2 (++++)
100.0	74.3	5.7	50	215.6	0.1202	3 (++++)
200.0	174.3	5.7	50	415.6	0.2202	Negative.
100.0	74.3	10.8	50	220.7	0.1227	++++
34.7	53.4	48.0	50	171.7	0.0982	3 (++++)
34.7	75.4	70.0	50	215.7	0.1202	++++
34.7	101.0	96.0	50	267.3	0.1460	++++
34.7	150.0	146.0	50	366.3	0.1955	++ to +++
34.7	175.0	170.0	50	415.3	0.2200	Negative.

that the effect of $[Na^+]$ or of $[K^+]$ is not due to any specific effect of these ions but rather to changes in the total ionic strength of the solutions.[2] In view of the well known depressant effect of potassium ion upon the heart the tolerance of the cartilage cells for potassium, even in high concentrations, is worthy of emphasis.

[2] In our experiments the interchange between $[Na^+]$ and $[K^+]$ was gross and no appreciable changes in calcification were noted when one or the other cation was used.

D. Effect of Magnesium on Calcification in Vitro.[3]

Blood serum contains only small amounts of magnesium. The normal figures for human blood serum are from 1.8 to 2.2 mg. per 100 cc. It would hardly be expected that an increase in the concentration of magnesium of only 1.8 mg. per 100 cc. should interfere with calcification. However, experimentally, we have found that calcification occurs most rapidly in the absence of magnesium. The introduction of magnesium either in the form of magnesium

TABLE III.

Effect of $[Mg^{++}]$ on Calcification in Vitro at pH 7.25 to 7.35 and 37–38°, and with Varying Amounts of Calcium and Phosphorus.

$[Na^+]$	$[Cl^-]$	$[Ca^{++}]$	$[Mg^{++}]$	$[PO_4^{=}]$	$[SO_4^{=}]$	Ca × P	$[Mg^{++}]$	Total mM concentration.	μ	Calcification.
mM	*mM*	*mM*	*mM*	*mM*	*mM*	*mg. per cent*	*mg. per cent*			
100	75	2.5	0.0	0.8	0.0	25	0.0	214.0	0.1139	Negative.
100	75	2.5	0.75	0.8	0.75	25	1.8	215.5	0.1169	"
100	75	2.5	0.0	0.96	0.0	30	0.0	214.16	0.1146	++++
100	75	2.5	0.75	0.96	0.75	30	1.8	215.66	0.1176	Negative.
100	75	2.5	1.5	0.96	1.5	30	3.6	217.16	0.1206	"
100	75	2.5	0.0	1.6	0.0	50	0.0	214.8	0.1175	2(++++)
100	75	2.5	0.75	1.6	0.75	50	1.8	216.3	0.1205	2(++++)
100	75	2.5	1.5	1.6	1.5	50	3.6	217.8	0.1235	Negative.
100	75	2.5	3.0	1.6	3.0	50	7.2	220.8	0.1295	"
100	75	2.5	1.5	1.9	1.5	60	3.6	218.1	0.1249	"
100	75	2.5	1.5	2.2	1.5	70	3.6	218.4	0.1262	2(++++)
100	75	2.5	3.0	2.2	3.0	70	7.2	221.4	0.1332	Negative.
100	75	1.75	0.0	2.25	0.0	49	0.0	228.7	0.1189	++++
100	75	1.75	0.75	2.25	0.75	49	1.8	230.2	0.1219	++++
100	75	1.75	1.5	2.25	1.5	49	3.6	231.7	0.1249	Negative.
100	75	1.75	3.0	2.25	3.0	49	7.2	334.7	0.1309	"
150	125	2.5	0.75	1.6	0.75	50	1.8	315.8	0.1705	++++
150	125	2.5	1.5	1.6	1.5	50	3.6	317.8	0.1735	Negative.
150	125	2.5	3.0	1.6	3.0	50	7.2	320.8	0.1965	"

[3] A preliminary report on the effect of magnesium on calcification appeared in the Howland Memorial Number, *Bull. Johns Hopkins Hosp.*, 1927, xli, 426.

chloride or magnesium sulfate, in excess of the normal concentration in serum, definitely inhibits the process. The inhibitory effect progressively increases as the magnesium concentration rises. The amount of additional magnesium which suffices to inhibit calcification is small as regards its effect on the ionic strength of the solution, as may be seen from Table III. It may be due to a specific effect of the divalent magnesium ion. It is possible that magnesium forms a slightly dissociable phosphate compound or that it influences the phosphate ion activity. This seems plausible from the consideration that the inhibitory effect of the magnesium ion can be overcome by suitable increments of inorganic phosphate. It is not unlikely that the magnesium ion exerts an injurious effect upon the cartilage cells themselves, thereby interfering with calcification even when physicochemical conditions are favorable.

E. Effect of Bicarbonate.

Solutions were made up in the usual way except that different quantities of bicarbonate were added to the various solutions. The pH was then adjusted by means of CO_2. These solutions consequently differed from one another as regards their content of both bound and free CO_2. When analyzed for CO_2 according to the method of Van Slyke and Neill (12), the concentration was found to vary from 20 to 44 mM; the average was 30 mM. Apparently within these limits of bicarbonate concentration and at the optimal pH, the concentration of bicarbonate does not materially affect the process of calcification *in vitro*.

F. Calcification in Calcium-Phosphate Solutions Containing Minimal Amounts of Other Electrolytes.

The observation that calcification occurs more readily at a total molal concentration lower than that of blood serum, led us to study calcification in solutions containing only calcium and phosphorus. A stock solution of H_3PO_4 was made so that 1 cc. of stock solution contains 5 mg. of phosphorus, and a calcium stock solution was prepared by adding 0.5 cc. of concentrated HCl to a solution containing 0.5 gm. of $Ca(OH)_2$, and making up the volume to a liter. To a liter flask containing about 500 cc. of water and 20 cc.

of indicator, the desired amounts of H_3PO_4 solution and 370 cc. of the calcium stock solution were added. The solution was made up to volume and the pH adjusted with CO_2. It was found that the pH of the solution could not be maintained unless 5 mM of $[Na^+]$ were added as NaOH. Thus the final solution contained theoretically and by analysis $[Ca^{++}]$ 2.5 mM, $[PO_4^{\equiv}]$ 1.6 mM, $[Na^+]$ 5 mM, $[Cl^-]$ 1.0 mM, and about 30 mM of $[HCO_3^-]$.

The first attempt yielded results in accordance with our expectation. Excellent calcification occurred in the provisional zone of calcification within 18½ hours, in solutions with a calcium and phosphorus product of 50. However, on repeating the experiments under similar conditions a few days later the results were

TABLE IV.

Calcifications in Solutions of Minimal Total Molar Concentrations and Varying Amounts of Calcium and Phosphorus, at pH 7.25 to 7.35 and 37–38°. $[Na^+]$ 5.0 mM, $[Cl^-]$ 1.0 mM, $[HCO_3^-] \pm 10$ mM.

$[Ca^{++}]$	$[PO_4^{\equiv}]$	Ca × P	Total mM concentration.	μ	Calcification.	Precipitation.	Remarks.
mM	*mM*	*mg. per cent*					
1.25	0.66	10	16.91	0.0123	Negative.	+	After ½ hr. incubation pH changed to about 6.9.
1.66	0.80	15	17.46	0.0144	"	+	
1.75	0.98	21	17.73	0.0154	"	+	
2.5	0.8	25	18.30	0.0161	"	+	
2.5	0.98	30	18.48	0.0169	"	+	
2.5	1.6	50	18.96	0.0197	"	+	

entirely negative. Subsequent attempts were similarly unsuccessful. After incubation a heavy precipitate appeared and the supernatant fluid became acid. However, no changes occurred in our control solutions containing the same amount of calcium and phosphorus and in addition other electrolytes to a total mM concentration of over 200. In these the pH remained constant, and no visible precipitates appeared even after several days incubation. This suggested that the presence of other ions prevented rapid precipitation of calcium salts. Consequently, the following experiments were carried out.

To a solution containing calcium and phosphorus prepared as

described, NaCl was added in increasing amounts. One series of flasks was used for calcification *in vitro*, and the other stoppered without introducing sections of bone. The results are given in Table IV. It is seen that when the product of the concentrations of calcium and phosphorus equals 50 and the concentration of $[Na^+]$ is 5 mM, precipitation occurred within half an hour and the reaction of the supernatant fluid became acid. When NaCl was added to the solution, rapid precipitation was prevented only when the concentration of this salt was increased to about 105 mM. Under these conditions the reaction and the concentration of the solution remained at optimum for the period necessary for calcification (18 to 20 hours).

TABLE V.

Effect of Increasing Total Molar Concentration on Calcification and Solubility of Calcium Salts. $[Ca^{++}]$ *2.5 mM*, $[PO_4^{\equiv}]$ *1.6 mM.*

$[Na^+]$	$[Cl^-]$	$[HCO_3^-]$	Ca × P	Total mM concentration.	μ	Calcification.	Precipitation.
mM	*mM*	*mM*	*mg. per cent*				
40.5	40.5	10.0	50	95	0.0572	Negative.	+
83.0	83.0	20.0	50	190	0.1047	+	Negative.
107.0	107.0	30.0	50	248	0.1342	++++	"
227.0	224.0	30.0	50	485	0.2527	Negative.	"

In the next experiment the effect of temperature on precipitation was determined. Solutions were prepared as above. Some were kept at room temperature, others were placed in an incubator at 38°, while a third group was first kept at a temperature of 34° for 5 hours and then at 36° for an additional 14 hours. It was noted that while those incubated at 38° formed a precipitate within half an hour, no change occurred in those kept at 34° or at room temperature. The results are given in Table V. On looking over our protocols we observed that our previous failures to obtain calcification *in vitro* in more than ten attempts and our first successful attempt coincided with higher and lower temperatures respectively. Calcification occurred at a temperature not higher than 36° and was entirely absent at 37° and 38° because of rapid precipitation.

G. Effect of Magnesium on Precipitation and Stability of Calcium-Phosphate Solutions.

The observation that the addition of small amounts of magnesium to optimal solutions inhibits calcification, presumably by combining with the phosphate and forming a slightly dissociable magnesium salt, and that less NaCl is needed to prevent precipitation when magnesium is also present in solution (Tables I and IV), suggested that magnesium may inhibit rapid precipitation of simple calcium-phosphate solutions at 37°. Consequently solutions were prepared containing [Ca^{++}] 2.5 mM, [$PO_4^{=}$] 1.6 mM (Ca × P = 50), [Na^{+}] 5 mM, [Mg^{++}] 0.75 mM (1.8 mg. per 100 cc.), [$SO_4^{=}$] 0.75 mM, and [Cl^{-}] 1.0 mM. The pH was kept between 7.25 and 7.35. One series was employed for calcification *in vitro* and the other was used for observing the formation of a precipitate. Table VI shows that the addition of 1.8 mg. of magnesium per 100 cc. of the solution, an amount equivalent to that found in blood serum, is capable of preventing the precipitation of calcium salts from solutions, long enough for calcification to occur. On the other hand similar solution without magnesium became acid within half an hour with the formation of a heavy precipitate. However, when the solutions were allowed to incubate too long or when the temperature was raised above 38°, 0.75 mM of magnesium failed entirely to prevent precipitation.

TABLE VI.

Effect of [Mg^{++}] on Precipitation and Solubility of Calcium Phosphate Incubated at 37° for 17 Hours. [Ca^{++}] 2.5 mM, [$PO_4^{=}$] 1.6 mM, [Na^{+}] 5.0 mM, [Cl^{-}] 1.0 mM, and [HCO_3^{-}] 10 mM.

Initial pH.	Final pH.	[Mg^{++}]	[$SO_4^{=}$]	Ca × P	Total mM concentration.	μ	Precipitation.	Calcification.
		mM	*mM*	*mg. per cent*				
7.25	7.25	0.75	0.75	50	18.1	0.0232	Negative.	++++
7.35	7.35	0.75	0.75	50	18.1	0.0232	"	++++
7.25	6.8	0.0	0.0	50	15.1	0.0202	++++	Negative.
7.35	6.9	0.0	0.0	50	15.1	0.0202	++++	"

SUMMARY AND CONCLUSION.

Shipley's observation on the calcification of rachitic cartilage when incubated in normal rat serum supplied a new method for the study of calcification. The remarkable localization of the *in vitro* calcification and its failure to occur when the cells have been injured by protoplasmic poisons at once differentiates this process from that which occurs in pieces of dead cartilage, whether *in vivo* or *in vitro*, and proves that we are dealing with a process similar, at least in some respects, to that occurring *in vivo*.

Two sets of conditions may affect calcification: (1) conditions affecting the cartilage cells themselves, (2) changes in the physicochemical composition of the solution bathing the cells. Hitherto the latter has been the point of attack. The composition of the solution has been modified in various ways and the effect upon *in vitro* calcification has been studied. We have shown that the reaction of the solution is one factor that determines calcification. There is an optimal reaction, that of normal blood serum. An increase of the total ionic strength of the solution whether produced by increased amounts of sodium chloride or potassium chloride inhibits calcification. This inhibition occurs at lower concentrations of sodium chloride or potassium chloride if the concentrations of calcium and phosphate are also lower. Calcification occurs more readily in the absence of magnesium. The inhibitory effect of magnesium can be overcome by the addition of suitable amounts of phosphate, the mechanism of which cannot be explained at present. This inhibitory effect may be due either to the toxic effect of magnesium ion upon cartilage cells, or the formation of an unionized magnesium compound.

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THE TRANSFORMATION OF CREATINE INTO CREATININE BY THE MALE AND FEMALE HUMAN ORGANISM.

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Since the publication of the classical paper of Folin (1906), in which he concluded that creatine and creatinine are independent of each other in metabolism, a number of articles have appeared indicating that, contrary to Folin's views, a limited amount of creatine may be transformed into its anhydride in the animal organism. (For a review of the literature see Hunter, 1922.) The most noteworthy contribution dealing with this problem is the recent paper of Benedict and Osterberg (1923). These authors have shown that in dogs the long continued oral administration of creatine leads to a very gradual increase in creatinine excretion which, after several weeks, may exceed the normal output by 22 to 33 per cent. This observation permits of no reasonable doubt that dogs can dehydrate creatine; but the remarkable feature is the fact that days or even weeks are required for the completion of the transformation.

In man no investigations of a similar sort have been reported. In experiments conducted several years ago, and involving the administration of large doses of creatine over brief periods of time, Rose and Dimmitt (1916) observed unmistakable increases in creatinine elimination. The paper of Benedict and Osterberg suggested to us the desirability of determining whether the reaction is as slow and difficult for the human organism to accomplish as it is for the dog. But we were especially interested in comparing the behavior of the male and female human subjects. Such a comparison should be of interest in connection with the well known fact that women are more subject to creatinuria than are men.

If the creatinuria of the female is due to a failure in creatine dehydration, this fact should be manifested following the prolonged administration of creatine. On the other hand, if the difference between the sexes is due to unlike powers of retention, this also should be capable of demonstration. With these points in mind, two prolonged experiments were carried out, one upon a male and the other upon a female. The results, which are outlined below, are so clear cut and agree so closely with the data secured by Bene-

TABLE I.
Composition of Diets.

	Male subject.	Female subject.
	gm.	*gm.*
Bread (whole wheat)	200	200
Butter	75	75
Eggs (raw weight)	100	100
Milk	1200	1200
Potato (boiled)	200	150
Beans (canned Lima, cooked)	125	125
Lettuce	100	100
Mayonnaise	20	20
Bran	25	25
Sugar	15	15
Jelly	40	40
Orange	300	300
Nitrogen content,* *gm.*	14.75	14.55
Energy value,* *calories*	2970	2920

* Most of the nitrogen and energy data are taken from Atwater, W. O., and Bryant, A. P., *U. S. Dept. Agric., Office Exp. Stations, Bull. 28* (Revised), 1906; but the milk, mayonnaise, jelly, beans, and bran were analyzed for total nitrogen.

dict and Osterberg upon dogs, that it seemed to us unnecessary to make additional experiments of this kind.

The subjects were normal graduate students in this laboratory, and were selected for the purpose in question because of their similarity in body weight. Both were subjected to exactly the same treatment as regards dosage and length of time of the creatine administration. The diets employed are shown in Table I. As will be observed, they are identical with the exception that the

male subject ingested a somewhat larger quantity of potatoes than did the female. The creatine was a commercial product which had been purified by repeated recrystallizations until entirely free of creatinine, and then dried *in vacuo*. Three such preparations were employed. Analyses showed the presence of traces of water of crystallization in each, for which allowance was made in calculating the creatine intake. As indicated in Tables II and III, the daily dosage of anhydrous creatine (present in 1 gm. quantities of the three preparations) amounted to 0.97, 0.95, and 0.96 gm. The creatine was taken in two equal amounts mixed with the food at the morning and noon meals. Great care was exercised in the urine analyses to insure the maintenance of identical conditions throughout. But for a few unavoidable exceptions the volumes were always made up to the same quantity. Total nitrogen was determined by the Kjeldahl-Gunning method, while total and preformed creatinine were estimated by the Folin (1914) procedures. In the latter, creatinine of known purity was employed as the standard. The subjects ingested the constant diets for several days preceding the collection of the first urines.

The results of the two experiments are detailed in Tables II and III. In each experiment the creatine administration was begun after two preliminary periods of 7 days each and was continued for seven periods of 7 days each. Five after periods followed during which no creatine was given. In the male subject (Table II), the remarkable fact is brought out that no unchanged creatine was excreted during the entire experiment. Perhaps this would not occur invariably in males, inasmuch as the power of retention might depend somewhat upon the degree of saturation of the tissues when the creatine administration was inaugurated. The creatinine output showed no appreciable increase until the third period of creatine feeding, and from that time slowly but steadily increased until the end of the first after period. Indeed, the average daily output was slightly larger for the first after period than for the preceding weeks during which creatine was administered. The maximum for a single day was on the last day of the first after period when the output amounted to 2.22 gm., or an increase over the average for the preliminary periods of 0.44 gm. (24.7 per cent).

During the second after period the creatinine began to decline

TABLE II.

Fate of Ingested Creatine.

Subject 1, Male.

This subject excreted no creatine as creatinine throughout the experiment.

Date.	Body weight.	Creatine ingested.	Urine.		Creatinine coefficient.	Remarks.
			Total N.	Creatinine.		
	kg.	*gm.*	*gm.*	*gm.*		
Feb. 28	72.7			1.82		First preliminary period.
Mar. 1				1.84		
" 2				1.80		
" 3				1.80		
" 4				1.76		
" 5				1.70		
" 6				1.75		
		0	12.15	1.78	24.5	Average of 7 days.
Mar. 7	72.3			1.74		Second preliminary period.
" 8				1.73		
" 9				1.77		
" 10				1.78		
" 11				1.80		
" 12				1.82		
" 13				1.73		
		0	12.14	1.77	24.5	Average of 7 days.
Mar. 14	72.3			1.72		First period of creatine administration.
" 15				1.74		
" 16				1.75		
" 17				1.68		
" 18				1.77		
" 19				1.72		
" 20				1.81		
		0.97*	12.19	1.74	24.1	Average of 7 days.
Mar. 21	72.1			1.81		Second period of creatine administration.
" 22				1.76		
" 23			Sample lost.			
" 24				1.75		
" 25				1.81		
" 26				1.80		
" 27				1.84		
		0.97*	11.81	1.80	25.0	Average of 6 days.

* Same amount for each day of period.

TABLE II—*Continued.*

Date.	Body weight.	Creatine ingested.	Urine.		Creatinine coefficient.	Remarks.
			Total N.	Creatinine.		
	kg.	*gm.*	*gm.*	*gm.*		
Mar. 28	72.7			1.84		Third period of creatine administration.
" 29				1.87		
" 30				1.89		
" 31				1.89		
Apr. 1				1.87		
" 2				1.81		
" 3				1.84		
		0.97*	11.23	1.86	25.7	Average of 7 days.
Apr. 4	72.2	0.97		1.85		Fourth period of creatine administration.
" 5		0.97		1.90		
" 6		0.95		1.77		
" 7		0.95		2.05		
" 8		0.95		1.99		
" 9		0.95		2.00		
" 10		0.95		1.92		
		0.96	11.92	1.93	26.7	Average of 7 days.
Apr. 11	72.8			1.88		Fifth period of creatine administration.
" 12				2.02		
" 13				1.98		
" 14				1.96		
" 15				1.98		
" 16				2.09		
" 17				2.11		
		0.95*	12.20	2.00	27.5	Average of 7 days.
Apr. 18	72.5			2.14		Sixth period of creatine administration.
" 19				1.95		
" 20				1.98		
" 21				2.01		
" 22				1.95		
" 23				2.01		
" 24				2.11		
		0.95*	11.91	2.02	27.9	Average of 7 days.

* Same amount for each day of period.

TABLE II—*Continued.*

Date.	Body weight.	Creatine ingested.	Urine.		Creatinine coefficient.	Remarks.
			Total N.	Creatinine.		
	kg.	*gm.*	*gm.*	*gm.*		
Apr. 25	72.4	0.95		2.10		Seventh period of creatine administration.
" 26		0.95		2.17		
" 27		0.95		2.05		
" 28		0.95		2.16		
" 29		0.96		1.98		
" 30		0.96		2.17		
May 1		0.96		2.03		
		0.95	11.17	2.09	28.9	Average of 7 days.
May 2	72.9			2.06		First after period.
" 3				2.10		
" 4				2.01		
" 5				2.17		
" 6				2.21		
" 7				2.21		
" 8				2.22		
		0	11.74	2.14	29.4	Average of 7 days.
May 9	72.4			2.17		Second after period.
" 10				2.13		
" 11				1.93		
" 12				1.94		
" 13				1.85		
" 14				2.02		
" 15				2.04		
		0	12.24	2.01	27.8	Average of 7 days.
May 16	72.2			1.95		Third after period.
" 17				2.01		
" 18				1.99		
" 19				1.88		
" 20				1.83		
" 21				1.97		
" 22				1.89		
		0	11.74	1.93	26.7	Average of 7 days.

TABLE II—*Concluded.*

Date.	Body weight.	Creatine ingested.	Urine.		Creatinine coefficient.	Remarks.
			Total N.	Creatinine.		
	kg.	*gm.*	*gm.*	*gm.*		
May 23	72.4			1.96		Fourth after period.
" 24				1.94		
" 25				1.88		
" 26				1.90		
" 27				1.75		
" 28				1.84		
" 29				1.75		
		0	11.55	1.86	25.7	Average of 7 days.
May 30	72.2			1.88		Fifth after period.
" 31				1.83		
June 1				1.91		
" 2				1.80		
" 3				1.75		
" 4			Sample lost.			
" 5				1.79		
		0	11.04	1.83	25.3	Average of 6 days.

and continued to diminish slowly and somewhat irregularly to the end of the experiment. On the last day of the fifth after period the output was practically identical with the average daily excretion of the fore periods.

The results of the experiment upon the female (Table III) manifest some interesting variations from the data secured with the male subject. During the preliminary periods, the female excreted small and variable amounts of creatine. With the beginning of the creatine feeding, no alteration in the creatine output was observed until the third period. It then began to increase quite rapidly and reached a maximum in the fourth period, when approximately one-third of that administered reappeared unchanged in the urine. During the last three periods of creatine feeding the average output remained fairly constant although the figures from day to day show rather wide fluctuations. On the 2nd day of the first after period the creatine excretion dropped to

TABLE III.

Fate of Ingested Creatine.

Subject 2, Female.

Date.	Body weight.	Creatine ingested.	Urine.			Creatinine coefficient.	Remarks.
			Total N.	Creatinine.	Creatine as creatinine.		
	kg.	*gm.*	*gm.*	*gm.*	*gm.*		
Feb. 28	72.9			1.25	0.09		First preliminary period.
Mar. 1				1.27	0.12		
" 2				1.27	0.08		
" 3				1.25	0.08		
" 4				1.25	0.07		
" 5				1.26	0.10		
" 6				1.25	0.08		
		0	11.97	1.26	0.09	17.3	Average of 7 days.
Mar. 7	72.1			1.26	0.05		Second preliminary period.
" 8				1.26	0.06		
" 9				1.26	0.05		
" 10				1.28	0.07		
" 11				1.30	0.03		
" 12				1.31	0		
" 13				1.26	0.07		
		0	11.53	1.28	0.05	17.8	Average of 7 days.
Mar. 14	72.7			1.25	0.06		First period of creatine administration.
" 15				1.26	0.07		
" 16				1.36	0.05		
" 17				1.31	0.05		
" 18				1.37	0.03		
" 19				1.28	0.05		
" 20				1.33	0.08		
		0.97*	10.89	1.31	0.06	18.0	Average of 7 days.
Mar. 21	73.3			1.33	0.05		Second period of creatine administration.
" 22				1.36	0		
" 23				1.37	0		
" 24				1.38	0.10		
" 25				1.37	0.08		
" 26				1.42	0.10		
" 27†							
		0.97*	11.47	1.37	0.06	18.7	Average of 6 days.

* Same amount for each day of period.

† Menstruation; urine not collected.

TABLE III—*Continued.*

Date.	Body weight.	Creatine ingested.	Urine.			Creatinine coefficient.	Remarks.
			Total N.	Creatinine.	Creatine as creatinine.		
	kg.	*gm.*	*gm.*	*gm.*	*gm.*		
Mar. 28†	73.3						Third period of creatine administration.
" 29†							
" 30				1.42	0.16		
" 31				1.40	0.27		
Apr. 1				1.41	0.19		
" 2				1.40	0.32		
" 3				1.38	0.36		
		0.97*	12.16	1.40	0.26	19.1	Average of 5 days.
Apr. 4	73.3	0.97		1.46	0.35		Fourth period of creatine administration.
" 5		0.97		1.42	0.30		
" 6		0.95		1.48	0.27		
" 7		0.95		1.41	0.40		
" 8		0.95		1.49	0.30		
" 9		0.95		1.48	0.43		
" 10		0.95		1.44	0.34		
		0.96	11.61	1.45	0.34	19.8	Average of 7 days.
Apr. 11	73.5			1.46	0.34		Fifth period of creatine administration.
" 12				1.49	0.30		
" 13				1.44	0.31		
" 14				1.50	0.29		
" 15				1.53	0.30		
" 16				1.55	0.26		
" 17				1.54	0.19		
		0.95*	10.95	1.50	0.28	20.4	Average of 7 days.
Apr. 18	73.4			1.52	0.20		Sixth period of creatine administration.
" 19				Sample lost.			
" 20				1.47	0.33		
" 21				1.48	0.22		
" 22				1.49	0.21		
" 23†							
" 24†							
		0.95*	11.90	1.49	0.24	20.3	Average of 4 days.

* Same amount for each day of period.
† Menstruation; urine not collected.

TABLE III—*Continued.*

Date.	Body weight.	Creatine ingested.	Urine.			Creatinine coefficient.	Remarks.
			Total N.	Creatinine.	Creatine as creatinine.		
	kg.	*gm.*	*gm.*	*gm.*	*gm.*		
Apr. 25†	73.4	0.95					Seventh period of creatine administration.
" 26		0.95		1.55	0.37		
" 27		0.95		1.53	0.33		
" 28		0.95		1.43	0.27		
" 29		0.96		1.56	0.33		
" 30		0.96		1.52	0.50		
May 1		0.96		1.46	0.22		
		0.95	12.39	1.51	0.34	20.6	Average of 6 days.
May 2	73.2			1.53	0.43		First after period.
" 3				1.50	0.05		
" 4				1.50	0.06		
" 5				1.48	0.03		
" 6				1.56	0		
" 7				1.59	0.06		
" 8				1.54	0		
		0	11.57	1.53	0.09	20.9	Average of 7 days.
May 9	73.8			1.52	0.09		Second after period.
" 10				1.44	0		
" 11				1.49	0		
" 12				1.46	0		
" 13				1.57	0		
" 14				1.50	0		
" 15				1.53	0		
		0	12.10	1.50	0.01	20.3	Average of 7 days.
May 16	73.8			1.46	0		Third after period.
" 17				1.46	0		
" 18†							
" 19†							
" 20†							
" 21				1.41	0		
" 22				1.38	0		
		0	11.89	1.43	0	19.4	Average of 4 days.

† Menstruation; urine not collected.

TABLE III—*Concluded.*

Date.	Body weight.	Creatine ingested.	Urine.			Creatinine coefficient.	Remarks.
			Total N.	Creatinine.	Creatine as creatinine.		
	kg.	*gm.*	*gm.*	*gm.*	*gm.*		
May 23	73.4			1.38	0		Fourth after period.
" 24				1.40	0		
" 25				1.42	0		
" 26				1.38	0		
" 27				1.37	0		
" 28				1.42	0		
" 29				1.36	0		
		0	12.73	1.39	0	18.9	Average of 7 days.
May 30	73.4			1.48	0		Fifth after period.
" 31				1.40	0		
June 1				1.41	0		
" 2				1.35	0		
" 3				1.34	0		
" 4				1.33	0		
" 5				1.43	0		
		0	11.37	1.39	0	18.9	Average of 7 days.

its normal level, and after the 1st day of the second period failed to appear again in the urine. The latter fact is peculiar and unexpected in view of the almost constant excretion of small amounts during the preliminary periods.

The urinary creatinine showed a tendency to rise during the 1st week of creatine administration, and the increase became unmistakable during the 2nd week. The output continued to rise until the fifth period, and remained fairly constant from that time until the end of the second after period. As in the male subject, so in this individual, the maximum daily excretion was secured during the first after period when on the 6th day it amounted to 1.59 gm., or an increase of 0.32 gm. (25.2 per cent) over the average output for the preliminary periods. Following the withdrawal of creatine from the ration, the excretion of creatinine diminished to the end of the experiment, when it was almost down to the level of the fore periods.

In accordance with the procedure of Benedict and Osterberg we have calculated the percentage of the retained creatine which was transformed into creatinine by the two subjects. Inasmuch as neither of our subjects manifested decided alterations in body weight we have thought it advisable to compute the expected creatinine on the basis of the amounts actually excreted during the preliminary periods, rather than on the basis of the creatinine coefficients. A summary of the calculations in the two experiments

TABLE IV.

Percentage Recovery of Administered Creatine, and Percentage Transformation of the "Retained Creatine" into Creatinine as Calculated from Tables II and III.*

Figures for creatine are expressed as creatinine.

Subject.	Total creatine given.	Total creatine excreted as such.†	Total creatine expected.	Extra creatine.	Total creatine retained.	Total creatinine excreted.†	Total creatinine expected.	Extra creatinine.	Retained creatine excreted as creatinine.	Total recovery of administered creatine.
	gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*per cent*	*per cent*
1, male.	40.58	0	0	0	40.58	187.3	173.9	13.4	33.0	33.0
2, female.	40.58	12.7	6.7	6.0	34.58	138.7	124.1	14.6	42.2	50.8

* In accordance with the procedure of Benedict and Osterberg (1923) we have used the term "retained creatine" to denote the creatine not excreted as such.

† In calculating the total excretion of creatine and creatinine allowance was made for the days in which, because of accidental loss or the menstrual cycle, the urines were not collected. In each instance of this sort the figure representing the average output for the other days of the same period was employed in the calculation.

is presented in Table IV. As will be observed, 33.0 and 42.2 per cent of the retained creatine were transformed into creatinine in the male and female subjects respectively. These figures are rather close to those secured by Benedict and Osterberg in dogs, which manifested transformations of 29.1 to 34.2 per cent. The dosage of creatine in our experiments was relatively much smaller than that employed by Benedict and Osterberg. The latter administered 37.5 to 43.0 mg. (expressed as creatinine) per kilo of body weight per day. In our subjects, the daily dosage amounted

to 11.4 mg. per kilo. Thus it is rather striking that the two series of investigations involving such widely different dosages should show such closely agreeing values for creatine dehydration. Benedict and Osterberg suggest that creatine is metabolized in two or more ways, and that from 3 molecules of creatine only 1 molecule of creatinine results. The present investigation appears to ocnfirm this opinion.

Our data indicate quite clearly that the creatinuria of females is not associated with an inability to transform creatine into creatinine. Perhaps the fact that the female excreted a somewhat larger amount of extra creatinine than the male is not significant; but at least she was just as effective in accomplishing the transformation as was the subject of opposite sex. On the other hand, the female eliminated 6.0 gm. of unchanged creatine in excess of the expected amount, while the male excreted none. The total recovery of administered creatine amounted to 50.8 per cent in the female as compared with 33.0 per cent in the male. This fact suggests that females may be less efficient than males in storing or metabolizing that portion of the creatine which does not yield creatinine. If this conception is correct, one might expect to observe the excretion of creatine in women under circumstances which do not induce creatinuria in men. Perhaps the intermittent excretion of creatine by females and the creatinuria of high protein diets may be explained upon this basis.

The body weights of our subjects do not indicate that creatine ingestion exerts an influence upon nitrogen retention in the human organism such as was observed by Benedict and Osterberg in dogs. The weight of the male was quite constant throughout the experiment. The female showed a slight gain, but scarcely enough to justify the assumption that the administered creatine was instrumental in the change. It must be recalled, however, that the dosage employed by Benedict and Osterberg was approximately 3.5 times as large as that used by us. This difference may account for the greater influence upon nitrogen retention observed by the former investigators.

SUMMARY.

1. Long continued feeding experiments have shown that male and female human subjects possess the power of transforming crea-

tine into creatinine to approximately the same degree. This finding indicates that the creatinuria of women is not to be attributed to a failure in the conversion of creatinine into its anhydride.

2. Approximately one-third of the retained creatine was recovered as creatinine. This would seem to indicate, as Benedict and Osterberg suggest, that creatine is metabolized by two or more methods only one of which yields creatinine.

3. In the female, creatine reappears unchanged in larger amounts following its oral administration than in the male subjected to the same dosage. This suggests that the powers of retention and storage of creatine, or the ability to catabolize it by methods which do not yield creatinine, may be less effective in women than in men. Obviously, our data upon a single subject of each sex are not sufficient to justify one in making a positive assertion in this regard.

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THE PRODUCTION OF GLUCONIC ACID BY THE PENICILLIUM LUTEUM-PURPUROGENUM GROUP.

II. SOME OPTIMAL CONDITIONS FOR ACID FORMATION.*

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Within the last 4 years, several investigators (1) have noted the formation of mixtures of gluconic, citric, and oxalic acids in varying quantities, when *Aspergillus niger* and strains of the so called *Penicillium glaucum* were grown on sucrose and glucose solutions. They found that the ratio of the quantities of the different acids produced was widely changed by varying the experimental conditions; these influences were so pronounced that the oxidation could be almost completely directed to one of the three acids as the predominant end-product. In a previous paper of this series (1), the fact was announced that a certain strain of the *Penicillium luteum-purpurogenum* group (*Penicillium purpurogenum* var. *rubri-sclerotium* Thom, No. 2670), when cultured on glucose solutions, formed large quantities of gluconic acid to the exclusion of other acids which usually result from mold fermentations.

As it seemed unlikely that the exact conditions for the exclusive formation of gluconic acid by our organism had been accidentally used, it was thought advisable to vary several of the experimental factors so as to bring about the maximum production of acid and at the same time ascertain whether other acids were formed under these changed conditions. To this end experiments were devised whereby the temperature, concentration of glucose, and inorganic nutrient media were varied, and the results in each case were noted. The following methods were used.

* Read before the Section of Biological Chemistry of the American Chemical Society, September 5–12, 1927, Detroit, Michigan.

† Contribution 147.

Experimental Procedure.—All the experiments recorded were conducted in modified Erlenmeyer flasks made of Pyrex glass and having a capacity of 3 liters. They were specially designed some years ago for work of this type; the shape was such as to give the maximum surface area for the volume of solution employed and the necks were wide to facilitate cleansing as well as to permit a good diffusion of air. These flasks were stoppered with moderately hard rolled cotton plugs. 1000 cc. of culture solution were used throughout, giving a ratio of surface area to volume of 0.28. The solutions were sterilized at a pressure of 15 pounds of steam for 15 minutes. The period of culture was 14 days. In all experiments except those stated as otherwise, the inorganic nutrient salts employed were as follows:

	Gm. per liter of glucose solution.
$MgSO_4 \cdot 7\ H_2O$	0.5 (0.0049 per cent Mg, 0.0065 per cent S).
KCl	0.1 (0.0052 " " K).
K_2HPO_4	0.1 (0.0046 " " " 0.0017 per cent P).
$NaNO_3$	1.0 (0.016 " " N).

This salt solution was arbitrarily chosen from among the many such solutions that may be employed because of its low nitrogen content, which apparently favors production of acid by fungi cultured on sugar solutions (2–5). The cultures were made at a temperature of 25° except in experiments performed to determine the effect of temperature on the production of acid. The concentration of commercial glucose was 200 parts per 1,000 parts of solution, except when stated otherwise. This glucose was approximately 91.5 per cent pure and the remainder was almost entirely water. All yields were calculated on the basis of pure glucose. Cultures of the fungus were maintained on wort extract-agar slants, new transfers being made at approximately 15 day intervals.

Treatment of Fermented Culture Liquors.—The fermented solution was filtered, and the mycelium[1] was squeezed out on a Buchner funnel and washed with a small quantity of hot distilled water. The volume of the solution was noted, and samples were withdrawn for the determination of acid and glucose. The mycelium was then thoroughly rinsed with hot distilled water

[1] Mycelium and mycelia in this paper are used to designate the felt of fungus growth developing on the surface of the liquid substratum whether this felt is sporulating or vegetative.

and placed in the oven for 3 days at 80°, after which it was weighed to the nearest gm. The acid was in most instances recovered as the calcium or barium salt.

A modification of the Denigés test (6) was used for the detection of citric acid. Approximately 900 cc. of the culture solution were treated with an excess of calcium carbonate, heated to boiling, and filtered while hot. Sufficient sulfuric acid was added to the residue to produce a slightly acid reaction. The calcium sulfate was removed by filtration, and the entire filtrate, which contained the greater part of the citric acid in the culture solution, was tested according to the method of Denigés.

The culture solution was tested for oxalic acid by neutralizing a portion of the solution with ammonium hydroxide and adding calcium chloride. If present it would also be detected very easily in the modification of the Denigés test referred to above.

Determination of Gluconic Acid.—The quantity of this acid was determined by titration of the culture solution with 0.1 N sodium hydroxide at room temperature. Excess alkali was added, and the solution was maintained at a temperature of 50–55°. At this temperature it was found that any glucose present did not react with appreciable quantities of the sodium hydroxide and that the gluconic acid lactone was completely hydrolyzed to the acid in from 1 to 2 minutes. After 2 minutes at this temperature, the solution was titrated with 0.1 N sulfuric acid and the gluconic acid resulting from the hydrolysis of the lactone was added to the first titration. As a rule the lactone equalled from 5 to 10 per cent of the total quantity of gluconic acid. The total acid was then calculated in gm. of gluconic acid, since no citric or oxalic acids were found in appreciable quantities. To check these results several determinations of gluconic acid, as calcium gluconate, were made by neutralizing an aliquot portion of the culture liquor with calcium carbonate, heating to boiling, filtering, and precipitating the salt with 3 volumes of 95 per cent ethanol. The mixture was allowed to stand for 2 days to insure complete precipitation and was then filtered in a weighed Gooch crucible. The precipitate was washed with 60 per cent ethanol, after which it was dried to constant weight at 90°. The agreement between the quantity of acid calculated from the titration and the quantity actually recovered was satisfactory.

XUM

Determination of Glucose.—The glucose remaining unchanged in the culture liquor was determined according to the Benedict-Lewis method, the Willaman-Davison (7) modification being used. The yields were calculated as the ratio of glucose used for the production of gluconic acid to the total quantity of pure glucose originally in the solution.

Effect of Temperature.—The first variable considered was the temperature. Experiments were carried out at 20°, 25°, 30°, and 35°. Table I shows the effect of temperature on acid formation.

Temperatures in the vicinity of 25° seem to be the most favorable for the formation of acid. The production of acid at 20° proceeds at a rate appreciably slower than that at 25°. When

TABLE I.

Effect of Temperature.

Temperature.	Experiment No.	Duration.	Acid.	Yield.
°C.		*days*	*gm.*	*per cent*
20	1	14	98.2	49.3
	3	14	104.0	52.1
	5	21	102.2	51.3
25	1	14	115.0	57.5
	2	14	113.6	57.0
	4	14	111.5	56.0
30	1	14	41.6	20.9
	3	14	42.8	21.5
	4	14	40.6	20.4

maintained at these temperatures a thin continuous film of mycelium develops in about 3 days after inoculation; soon this is vigorous, grayish white, and has little or no sporulation. On the other hand, at 30° the initial development is more rapid; a continuous mycelium is formed in 2 days and follows the same course as at the lower temperatures until about the 8th day when the mycelium slowly begins to sink so that by the end of a fortnight it is completely submerged and inactive in the production of gluconic acid. The rate of production of acid decreases rapidly as the mycelium becomes submerged and the final yield of acid is much less than one-half that obtained at 25°. Experiments in which the organism was allowed to develop for the first 3 days at 30° and thereafter at

25° showed that approximately the same yields were obtained as when the entire course of the oxidation took place at 25°. At 35°, a film of continuous mycelium was developed in 2 days and improved slowly up to the 5th day after inoculation, after which it acquired a water-logged appearance and began to sink. The yield of acid at this temperature was very small.

Effect of Concentration of Glucose.—Attention was next devoted to the concentration of glucose at which the most satisfactory yields of acid could be obtained. Table II gives the results.

TABLE II.

Effect of Concentration of Glucose.

Commercial glucose per 1000 parts of solution.	Acid.	Sugar remaining.	Weight of mycelium.	Yield.
parts	*gm.*	*gm.*	*gm.*	*per cent*
100	41.6	36.0	5.0	41.8
100	40.5	36.9		40.7
150	66.7	46.8	5.0	44.6
150	73.4	44.6		49.1
200	108.0	45.9	6.0	54.2
200	113.0	39.6		56.7
250	128.6	73.8	7.0	51.6
250	129.7	61.2		52.0
300	105.8	140.4	6.0	35.1
300	103.0	145.8		34.5
350	74.8	200.7	5.0	23.4
350	71.1	205.2		20.4
400	41.8	280.8	4.0	10.5
400	50.1	279.0		12.6

There is little choice between the 20 per cent and 25 per cent glucose solutions, good percentage yields being obtained from both. The actual quantity of acid, however, is somewhat higher with the 25 per cent solution. The most vigorous mycelial growth took place on the 25 per cent cultures, but it was closely followed by the 30, 20, and 15 per cent, respectively. The mycelia were not nearly so vigorous on the 10 per cent solution as would have been expected; the tendency on this concentration of glucose seemed toward a weak and more or less water-logged mycelium resembling somewhat the mycelia on the 40 per cent concentrations.

The development of the fungus on the 35 per cent and 40 per cent solutions was much slower than on the others, and consequently the production of acid began at a later period.

Effect of Salts in the Solution.—In search for the inorganic salt solution most efficient as a nutrient, it was immediately found that the potassium chloride could be entirely eliminated from the formula since sufficient potassium was available in the dipotassium phosphate. In fact a slight increase in acid production was observed in every case where the potassium chloride was absent. The elimination of the dipotassium phosphate or magnesium sulfate from the culture solutions resulted in the formation of extremely scanty mycelia and no production of acid. When sodium nitrate was excluded, no growth occurred. The fact that development of the organism was so greatly inhibited under those

TABLE III.

Variation in Content of Magnesium ($MgSO_4 \cdot 7\ H_2O$).

Mg	Acid.	Sugar remaining.	Weight of mycelium.	Yield.
per cent	*gm.*	*gm.*	*gm.*	*per cent*
0.00098	97.2	58.2	5.0	48.0
0.00245	108.3	48.6	7.0	53.0
0.0073	110.0	36.7	7.0	55.0
0.0098	103.7	48.6	7.0	51.5

conditions is a good indication of the relatively high purity of the commercial glucose employed throughout this work.

The first salt to be varied in concentration was the magnesium sulfate. The results are given in Table III. As will be noted, no great difference in production of acid was found by varying the percentage of magnesium over wide ranges. It was observed, however, that when the concentration of this element fell below about 0.0024 per cent a distinct decline in the yield of acid occurred. This observation was confirmed in several other experiments.

The next element, the concentration of which was varied, was phosphorus. For this purpose disodium phosphate was used, and potassium chloride was added in sufficient quantity to maintain the usual percentage of potassium. The maximum yield of acid was obtained, as will be seen from Table IV, at a concentration of 0.00086 per cent P; this quantity was about one-half the quan-

tity of phosphorus in the standard culture medium. When the concentration fell below 0.00043 per cent, the yield of acid declined sharply, and the mycelium took on an entirely different appearance. Phosphorus starvation caused the development of an extremely thin though durable mycelial felt having a decided greenish to greenish yellow tint on the surface. In these extremely low phosphorus cultures Denigés' tests for citric acid gave slightly positive results. It was also repeatedly observed that as the

TABLE IV.

Variation in Content of Phosphorus ($Na_2HPO_4 \cdot 12\ H_2O$).

P	Acid.	Sugar remaining.	Weight of mycelium.	Yield.
per cent	*gm.*	*gm.*	*gm.*	*per cent*
0.000017	17.2	124.0	1.0	8.6
0.00017	31.0	119.0	1.0	15.5
0.00043	92.6	65.8	3.0	46.5
0.00086	125.9	40.0	4.0	63.2
0.00172	104.3	46.5	4.0	52.3
0.0034	101.1	52.5	5.0	50.8
0.0172	88.0	75.0	5.0	44.2

TABLE V.

Variation in Content of Phosphorus (H_3PO_4).

P	Acid.	Sugar remaining.	Weight of mycelium.	Yield.
per cent	*gm.*	*gm.*	*gm.*	*per cent*
0.000017	16.8	159.2	1.0	8.4
0.00086	126.9	41.9	4.0	63.7
0.0017	123.0	44.7	5.0	61.7
0.0034	113.1	44.8	5.0	56.8

concentration of phosphorus was increased beyond a certain point the acid production decreased distinctly, but in all cases the mycelia appeared more vigorous and were somewhat heavier.

These observations were checked, phosphoric acid being used as a source of phosphorus. As shown in Table V, corresponding data were obtained for each concentration studied, indicating that the changes observed could not be due to an increase in concentration of sodium, as was first thought.

The concentration of potassium was varied over a considerable

range and was supplied in the form of potassium chloride. Below 0.00026 per cent K the mycelia were underdeveloped and thin and had a slightly water-logged appearance, and the yield of acid decreased considerably (Table VI). As in the case of the phosphorus, the quantity of acid formed decreased slightly but consistently above a certain concentration of potassium chloride.

Variation in the concentration of nitrogen had a decided effect on the yield of acid, as will be seen from Table VII. Below 0.008

TABLE VI.

Variation in Content of Potassium (KCl).

K	Acid.	Sugar remaining.	Weight of mycelium.	Yield.
per cent	*gm.*	*gm.*	*gm.*	*per cent*
0.000026	28.1	146.2	2.0	14.1
0.00026	129.4	30.0	5.0	64.9
0.00052	128.9	30.0	6.0	64.7
0.0026	124.6	29.0	7.0	62.5
0.0052	119.8	39.5	7.0	60.1
0.0104	117.4	40.0	8.0	57.3

TABLE VII.

Variation in Content of Nitrogen ($NaNO_3$).

N	Acid.	Sugar remaining.	Weight of mycelium.	Yield.
per cent	*gm.*	*gm.*	*gm.*	*per cent*
0.0008	25.7	147.7	1.0	12.9
0.0016	43.6	125.6	2.0	21.9
0.008	112.6	51.5	2.0	56.5
0.016	124.6	29.0	5.0	62.5
0.024	119.6	30.3	5.0	60.0
0.032	92.0	61.5	6.0	46.2

per cent N, in the form of sodium nitrate, the formation of acid declined and the mycelia became thin. As the concentration passed beyond 0.024 per cent N, the yields declined and the mycelia in all cases became extremely vigorous and formed a comparatively thick pad.

In the experiments to determine the effect of the various concentrations of nitrogen on acid formation, ammonium salts were used. The results of these experiments were so unexpected that

a separate study is being made of the relation between the source of nitrogen and the formation of gluconic acid by this organism. Ammonium chloride, ammonium sulfate, ammonium nitrate, and ammonium carbonate were used in varying concentrations as nitrogen sources. In every case the production of acid and consumption of glucose were much less than that at corresponding concentrations of nitrogen when sodium nitrate was used, although a fairly vigorous mycelial development resulted in each case. Slightly positive tests for citric acid have been obtained when the nitrogen is supplied in the form of ammonium salts. The results of these experiments might possibly have an important bearing on a number of the theories now advanced to explain the process of the oxidation of glucose to the various organic acids.

After consideration of the data accumulated in the experiments on the relation of inorganic nutrient salts to the production of acid, a standard nutrient salt solution of the following composition was prepared.

	Gm. per liter of glucose solution.
$MgSO_4 \cdot 7\ H_2O$	0.25 (0.00245 per cent Mg, 0.00325 per cent S).
KCl	0.05 (0.0026 " " K).
$Na_2HPO_4 \cdot 12\ H_2O$	0.1 (0.00086 " " P).
$NaNO_3$	1.0 (0.016 " " N).

Of the many nutrient solutions employed, this is the most economical and gives the most consistent results. The percentage yields from a great number of experiments ranged from 55 to 62.5 per cent of the theoretical quantity of gluconic acid to be expected from the glucose originally present. The weights of the mycelia obtained in this series of experiments were comparatively low, and the quantity of sugar oxidized to carbon dioxide was not unduly large. The unchanged glucose remaining in the culture solutions averaged 40 gm.

The rate of production of the gluconic acid has been studied in a preliminary way. Portions of a large number of culture solutions have been titrated at varying intervals after inoculation. When the quantity of 0.1 N alkali required to neutralize 10 cc. of the culture liquor is plotted against the time in days after inoculation, a curve representing the velocity of the oxidation is obtained. A typical example of such a curve is indicated by the continuous line in Fig. 1. The strong resemblance to the average growth

curve will be noted immediately. The rate of oxidation appears to be highest between the 5th and 9th days after inoculation and is almost constant during that period. After the 9th day the velocity of the reaction invariably declines; this decline may be owing to one factor or a combination of several factors. Hydrogen

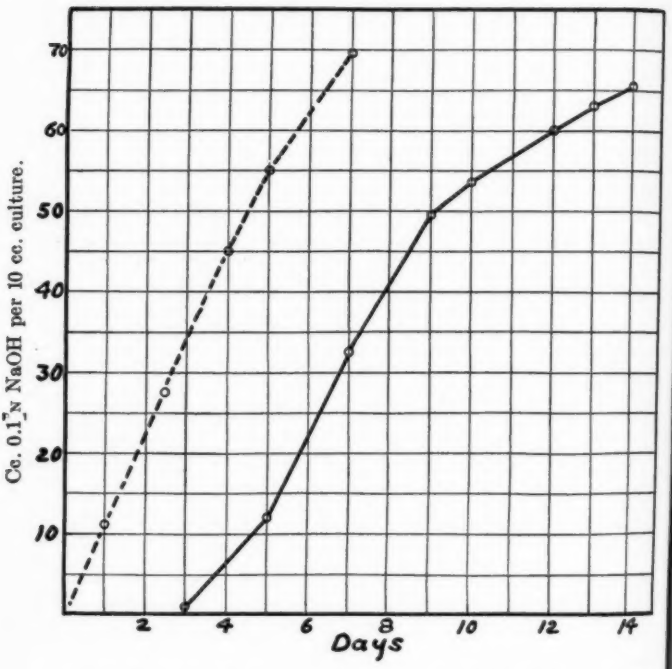

FIG. 1. The solid line indicates primary production of gluconic acid; the dotted line, secondary production of gluconic acid by the same mycelium.

ion concentration, the decrease in the quantity of glucose present, and the formation of by-products toxic to the oxidizing enzyme may be mentioned as possible contributing causes to the rather sharp break in the velocity curve.

It has been found that when the gluconic acid content has

reached the maximum, if the culture liquor is replaced with fresh sterile glucose solution containing no inorganic nutrients, the oxidation proceeds at once at a rapid rate. The curve representing the second oxidation is indicated by the broken line in Fig. 1. The break in rate of oxidation is not so pronounced in this case, and the yields are somewhat higher. Further studies of this fermentation are being made in an effort to bring about the oxidation at its maximum rate for a longer period, with a view to obtaining within a reasonable time the maximum yield of gluconic acid and the possible elimination of residual glucose.

In conclusion the authors wish to express their appreciation of the advice and assistance given in this work by Dr. Charles Thom and Dr. Margaret B. Church of the Bureau of Chemistry and Soils.

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OBSERVATIONS UPON THE ENZYME ASPARAGINASE.*

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The existence, in certain animal and vegetable tissues or juices, of more or less specific amide-splitting enzymes was demonstrated in 1902 by Gonnermann (1). His observations failed to include the biologically important amide asparagine; and the first to observe a fermentative hydrolysis of this substance into aspartic acid and ammonia was Lang (2). Lang showed that not only asparagine but also glutamine—both amides of amino acids—can be completely hydrolyzed by suspensions of beef liver; and that the first at least is split also by kidney, spleen, and testis, and perhaps even by the mucous membrane of the intestine. Confirmatory results with the tissues of the horse and the pig were reported a few years later by von Fürth and Friedmann (3), who concluded that the capacity to hydrolyze asparagine is possessed by all animal organs in an approximately equal degree. According to the more recent work of Clementi (4) the distribution of asparaginase in the animal kingdom is less universal than this conclusion would imply. Clementi found the enzyme present indeed in practically all the tissues of herbivorous animals; but in such omnivores as the pig and the rat it was strictly confined to the liver, while none of the organs of carnivorous mammals, of amphibians, of reptiles, of the monkey, or of man gave any evidence whatever of its existence. Clementi drew the deduction that the presence of asparaginase in animals is a specific biochemical adaptation to the diet. An observation which falls somewhat out of line with this generalization is that of Maeda (5), who reported that an extract of human placental tissue will liberate ammonia from asparagine. Maxa (6), it may be added, failed to find asparaginase in the testis of the bull, where its presence had been asserted by Lang.

About the same time that Lang observed the action of animal tissues upon asparagine Shibata (7) showed that it is slowly and incompletely hydrolyzed by the dried and powdered or acetone-treated mycelium of *Aspergillus niger*. A similar result was obtained by Dox (8) with another mould, *Penicillium camemberti*. Other vegetable sources of an asparagine-

* The experimental data of this paper are taken from a thesis submitted by W. F. Geddes in partial fulfilment of the requirements for the degree of Master of Arts in the University of Toronto.

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splitting enzyme have been indicated or established by the work of Nawiasky (9) on bacteria, that of von Fürth and Friedmann (3), Kurono (10), and Effront (11) on yeast, and that of Kiesel (12) and Butkewitsch (13) on higher plants. In spite therefore of negative results obtained by Pringsheim (14) and Dieter (15) with yeast, it would seem certain that asparaginase has a fairly wide distribution not only in the animal but also in the vegetable kingdom.

In what degree the action observed upon asparagine is specific, and to what extent, if at all, other amides are subject to enzymatic hydrolysis, are questions upon which the conflicting evidence available has not yet yielded a decision.

The present study of asparaginase was undertaken as a preliminary to an attempt to utilize it in a quantitative determination of asparagine. For such a purpose it was necessary, we decided, to possess a preparation, moderate quantities of which should be capable of decomposing quantitatively within 24 hours at least so much asparagine as would yield 7 mg. of ammonia nitrogen. Much of the work hitherto reported has dealt with experiments extending over several days or even weeks, and has been carried out with extracts evidently much less active than such a requirement would demand. Our first endeavor therefore was to find adequate methods for the extraction and concentration of the enzyme. Its separation from other enzymes and from inert material was regarded as an object less immediately urgent, although not to be lost from view. A reasonably active preparation having been secured, it was necessary next to obtain some precise knowledge concerning the course of its action and the conditions of its activity. We studied therefore the influence upon it of varying hydrogen ion concentration, and made some observations upon the velocity curve of the enzymatic reaction which it promotes. In further experiments we settled, as far as our own preparations were concerned, the question of specificity, and touched, although only incidentally, upon those of the stability and the distribution of asparaginase.

1. Preparation of Active Asparaginase Solutions from Yeast.

Among possible sources of asparaginase the one which appeared to be on the whole the most promising and convenient was brewers' yeast, and the experiments first to be described were

undertaken with the object of determining whether this material actually contains the enzyme, and how the latter, if present, may be most efficiently extracted.

(*a*) *Comparison of Different Methods of Extraction.*

Bottom yeast, fresh from the brewery,[1] was placed in 1 gallon lots in large carboys. These were filled with cold tap water, shaken well, and allowed to stand till the yeast had settled. The supernatant liquid was siphoned off and the process of washing repeated three or four times. The yeast was then transferred to large Buchner funnels, and sucked as dry as possible; and the nearly uniform product so obtained was used in making the several preparations described below.

Preparation 1. Press Juice.—200 gm. of the fresh yeast cake were ground with fine quartz in a mortar, and kieselguhr was stirred in till the mass appeared dry. The juice was then obtained by subjecting the whole to a pressure of 400 atmospheres in a Buchner press.

Preparation 2. Water Extract of Fresh Yeast.—200 gm. were mixed with 300 cc. of water and 25 cc. of toluene, and allowed to stand, with frequent stirring, for 3 days in the ice chest. The extract was then freed from yeast cells by centrifugation and filtering.

Preparation 3. Glycerol Extract of Fresh Yeast.—This was prepared in the same way as the water extract, except that 50 per cent glycerol was used in place of the water.

Preparation 4. Water Extract of Unground Dried Yeast.—A portion of the fresh yeast cake was spread thinly on glass plates, and dried rapidly in a blast of air at ordinary temperature. In this process it lost 64 per cent of its weight. The quantity of dry yeast corresponding to 200 gm. of fresh yeast was therefore 72 gm. Half this amount, 36 gm., was extracted as before with water; but in order that the proportion of yeast solids to water should not be altered the quantity of water used was 214 (150 + 64) cc.

Preparation 5. Glycerol Extract of Unground Dried Yeast.—

[1] We are indebted to O'Keefe's Beverages, Ltd., of Toronto for frequent and generous supplies of this material.

This was made in the same way as the previous extract with the single substitution of 50 per cent glycerol for water.

Preparation 6. Water Extract of Ground Dried Yeast.—100 gm. of air-dried yeast were ground in a ball-and-pebble mill, both steel balls and quartz pebbles being used, until a test portion, suspended in water and examined under the microscope, showed no remaining intact cells. This required grinding for from 24 to 48 hours. The powder obtained was extracted with water in the same proportion and by the same procedure as for Preparation 4.

Preparation 7. Glycerol Extract of Ground Dried Yeast.—Another lot of the same powder was extracted with 50 per cent glycerol.

Preparation 8. Alcoholic Extract of Ground Dried Yeast.—36 gm. of the powder were extracted under the usual conditions with 214 cc. of 30 per cent (by volume) ethyl alcohol. The yeast was then removed by centrifugation and filtration. The filtered extract was allowed to stand for some days in the ice chest. During this interval it deposited a heavy precipitate (Preparation 8 a), leaving a clear supernatant liquid (Preparation 8 b).

Preparation 9. Water Extract of Alcohol-Extracted Ground Yeast.—The residue of yeast powder left in Preparation 8 was suspended in 214 cc. of water, and extracted in the ice chest for 3 days more.

The activity of these various extracts was determined and compared in the following way. 50 cc. of M KH_2PO_4 were mixed with 50 cc. of water and 100 cc. of M NaOH, producing an alkaline phosphate solution of 0.25 M concentration and pH about 9.2. To 10 cc. of this solution, introduced into a 25 cc. volumetric flask, were added 5 cc. of an approximately 1.5 per cent solution of asparagine[2] and 5 cc. of the extract to be tested. The flask was then filled to the mark with water, and its contents thoroughly mixed. With the least possible delay 15 cc. (three-fifths of the whole) were thereupon transferred to a large Pyrex test-tube (200 × 30 mm.), treated with 0.5 cc. of toluene, and set in a

[2] Different samples of asparagine employed contained varying amounts of water of crystallization and (sometimes) ash. Solutions were therefore made up in a manner only roughly quantitative, and their actual content of asparagine was estimated from nitrogen determinations.

water bath of which the temperature was maintained at 30° (±0.1°). The residue of the mixture was used for the electrometric determination of its pH, which was found to lie in the different instances somewhere between 7.4 and 7.8. At the end of a period of 20 hours the test-tube was removed from the water bath, an equal quantity of saturated potassium carbonate solution was added, and the liberated ammonia was determined by aeration into 0.02 N sulfuric acid and back titration in the usual way. With two of the extracts—Preparations 2 and 3 prepared from fresh yeast—additional experiments were run to ascertain whether their activity was liable to deteriorate upon standing. For this purpose the unused residue of each of these extracts was removed from the ice chest to a thermostat at 35°, and at 3 day intervals thereafter was tested upon asparagine in the manner which has just been described. Each and every experiment was accompanied by appropriate controls, by which were determined (1) the appreciable quantities of ammonia usually introduced with, or developing in, the enzyme extract and (2) the traces sometimes present in the original asparagine solution or, possibly, liberated from it during aeration by the potassium carbonate alone. The asparagine solution was also subjected to a Kjeldahl determination of its total nitrogen, the result of which showed that the incubated fraction of each mixture must have contained 4.02 mg. of amide nitrogen.

Every determination, including the blanks, was carried out in duplicate; but as the duplicates always showed excellent agreement, it may suffice to report the average results. These are exhibited side by side in Table I; and since in the preparation of every extract (the press juice only excepted) the ratio of yeast solids to extracting liquid was the same, they are all (with the one exception noted) directly comparable. The comparison leads to the following conclusions.

1. Simple aqueous extracts of fresh living yeast possess a very limited power to hydrolyze asparagine. The activity of such preparations is indeed so slight as almost to corroborate the finding of Dieter (15), according to whom suspensions of living but non-growing yeast cells have no action at all upon asparagine.

2. Extracts considerably more active are readily obtained from yeast which has simply been dried (Preparations 4 and 5); but

TABLE I.

Action upon Asparagine of Different Preparations from Yeast.

Preparation No.	Material extracted.	Extracting agent.	Interval between preparation of extract and its use.	pH of digestion mixture.	0.02 N NH_3 found.		Amide N liberated in 20 hrs. at 30° (total = 4.02 mg.).	
					Controls.*	Main experiment.		
					cc.	cc.	mg.	per cent
1	Fresh yeast.	Press.	None.	7.81	1.51	5.06	1.00	24.8
2	" "	Water.	"	7.41	0.47	1.27	0.22	5.6
		"	3 days (at 35°).	7.70	2.40	2.99	0.17	4.1
		"	6 days (at 35°).	7.80	3.60	3.91	0.07	1.7
		"	9 days (at 35°).	7.92	5.27	5.24	0.00	0.0
3	Fresh yeast.	Glycerol.	None.	7.54	0.44	2.48	0.57	14.1
		"	3 days (at 35°).	7.67	1.32	3.28	0.55	13.6
		"	6 days (at 35°).	7.76	2.85	3.65	0.23	5.8
		"	9 days (at 35°).	7.81	3.83	4.13	0.08	2.1
4	Dried yeast, unground.	Water.	None.	7.46	0.75	2.90	0.60	15.0
5	" "	Glycerol.	"	7.47	0.66	3.71	0.86	21.3
6	Dried yeast, ground.	Water.	"	7.58	0.69	13.99	3.72	92.5
7	" "	Glycerol.	"	7.62	0.70	15.20	4.05	100.6
8a + 8b†	" "	Alcohol, 30 per cent.	3 days (at 3°).	7.69	0.46	1.64	0.33	8.2
8b	" "	" "	" "	7.68	0.46	0.48	0.00	0.0
9	Residue from Preparation 8.	Water.	None.	7.67	0.64	1.05	0.11	2.8
8 + 9				7.59	1.17	2.62	0.41	10.3

* Including the enzyme blank, which is variable, and a constant asparagine blank of 0.05 cc.

† In this experiment the extract was shaken, so as to distribute the deposit through the supernatant liquid; in the next the latter only was employed.

even these hardly contain enough asparaginase to make them practically useful.

3. By far the most active preparations (Nos. 6 and 7) were those obtained from dried yeast ground to an impalpable powder. It would seem therefore that a really good yield of enzyme is possible only when the yeast cells are thoroughly disintegrated before extraction.

4. Although the preparation of a press juice involves the disintegration of the cells, the final product is nevertheless not specially active. The press juice was of course of smaller volume and higher concentration than the aqueous extract of fresh yeast; and accordingly, although it was bulk for bulk more active, it seems doubtful whether it contained an appreciably greater total quantity of enzyme. It would appear then that in its preparation much of the asparaginase that must have been expressed from the cells was afterwards lost. Adsorption upon kieselguhr suggests itself at once as a probable explanation. Direct evidence that such adsorption actually takes place will be furnished later.

5. Under all circumstances 50 per cent glycerol is a more efficient extracting agent than water.

6. Extracts, even of powdered yeast, made with 30 per cent alcohol are relatively inactive (Preparations 8a + 8b). Indeed, if such extracts are cooled and filtered from their less soluble constituents, they may show no activity whatever (Preparation 8 b). This inactivity is not due to a simple failure to extract the enzyme, for very little of the latter is left in the extraction residue (Preparation 9). Neither is it due to something like the separation of an enzyme from its coenzyme, for extract and residue combined (Preparations 8 + 9) show only the sum of their separate small activities. The conclusion seems inevitable that alcohol has a destructive effect upon asparaginase. Further evidence of such a destructive effect will appear in another connection.

7. The enzyme asparaginase, if present in sufficient concentration, can effect a quantitative decomposition of asparagine (see Preparation 7).

8. In the medium provided by the rather crude extracts here under consideration asparaginase undergoes a moderately rapid destruction. This destruction, as it occurs in weak preparations like Nos. 2 and 3, is complete in from 6 to 10 days at 35°. It is an

accompaniment, as the blank determinations show, of autolytic changes evidenced by a steady accumulation of ammonia, and is perhaps brought about by the action of the autolyzing enzymes.

Frequent repetition of the experiments, with or without variations in the proportion of yeast to extracting liquid, in the quantity of substrate, and in the ratio of substrate to enzyme, has served only to confirm these conclusions. It may therefore be taken as demonstrated that yeast is at least moderately rich in asparaginase, and that the best yields of that enzyme are obtained by disintegrating the yeast thoroughly in a suitable mill, and extracting it then with 50 per cent glycerol. Unfortunately glycerol extracts are exceedingly difficult to clarify either by filtration or centrifugation. For practical reasons therefore it has been found convenient to forego the advantages of the somewhat higher yield which glycerol affords, and to use almost exclusively aqueous extracts.

(*b*) *Effect of pH upon the Efficiency of the Extraction.*

When yeast powder is extracted with water or glycerol, the extract, as we found, always has a pH in the neighborhood of 6. Some observations made in another connection suggested that the efficiency of the extracting process might be greater at a less acid reaction. To test this possibility we performed the following experiment. Three 25 gm. lots of dried and ground yeast were treated respectively with 150 cc. of water (Preparation 10), a mixture of 140 cc. of water with 10 cc. of N NaOH (Preparation 11), and a mixture of 135 cc. of water with 15 cc. of N NaOH (Preparation 12). Extraction was allowed to proceed in each case for 3 days, and the extracts, having been centrifuged and filtered, were first submitted to a determination of their pH. Mixtures were then prepared, each of which contained (1) 10 cc. of a 1.5 per cent solution of asparagine, (2) 10 cc. of a 0.25 M phosphate buffer so chosen as to give in each instance a final pH approximating 8, (3) a quantity ranging in different cases from 1 to 5 cc. of one or another of the three enzyme extracts, and (4) enough water to make a total volume of 25 cc. From each mixture 15 cc. were withdrawn for a determination, in the manner already described, of the asparagine hydrolyzed during 20 hours at 30°. The residual 10 cc. was used for the measurement of the pH at which

the hydrolysis was effected. Each determination was accompanied by appropriate blanks; and a nitrogen determination on the asparagine solution showed that in each fraction of the mixtures taken there must have been present 7.42 mg. of amide nitrogen.

The results of this experiment, which are exhibited in Table II, were sufficiently definite. So long as the quantity of extract used was submaximal (4 cc. or less), the one which accomplished in the allotted time the greatest amount of work was always the one

TABLE II.

Effect of pH on the Extraction of Asparaginase.

Preparation No.	pH during extraction.	Enzyme extract per 25 cc. of digestion mixture.	pH of digestion mixture.	0.02 N NH_3 found.		Amide N liberated in 20 hrs. at 30° (total = 7.42 mg.).	
				Controls.	Main experiment.		
		cc.		*cc.*	*cc.*	*mg.*	*per cent*
10	6.06	1	8.00	0.33	4.72	1.23	16.5
		2	8.12	0.67	11.97	3.16	42.6
		4	8.21	1.21	22.75	6.03	81.2
		5	8.16	1.47	28.37	7.53	101.5
11	7.00	1	8.08	0.37	9.54	2.57	34.6
		2	8.00	0.67	17.22	4.63	62.5
		4	8.12	1.26	27.26	7.28	98.1
		5	8.14	1.42	28.12	7.48	100.8
12	8.44	1	8.18	0.33	6.78	1.81	24.3
		2	8.22	0.62	12.98	3.46	46.6
		4	8.20	1.21	23.95	6.37	85.8
		5	8.16	1.47	28.17	7.48	100.8

prepared at an approximately neutral reaction (pH 7.00). 4 cc. of this neutral extract were actually all but sufficient to hydrolyze the asparagine completely; while with each of the others, prepared respectively at pH 6.06 and 8.44, the smallest quantity which produced this effect was 5 cc. Evidently slight degrees of either acidity or alkalinity have a destructive influence upon the enzyme, or in some other way exert a disadvantageous effect upon the process of extraction. The latter therefore is best carried out at the neutral point.

2. Attempts at Concentration and Purification of Asparaginase.

In the experiments recorded in Table II the maximum amount of yeast extract present in any of the 15 cc. fractions analyzed was 3 cc. In every instance this was sufficient to decompose quantitatively within 24 hours an amount of asparagine containing 7.42 mg. of amide nitrogen. The crude extracts of these experiments possessed therefore already that degree of potency which our ultimate object required. Unfortunately extracts from other samples of ground yeast have not always shown so great an activity. We have been forced therefore to seek some method which would make possible the concentration of the enzyme, once extracted, into reasonable volumes, and which might perhaps at the same time effect its partial separation from inactive admixtures.

Among those methods of concentration which have been more or less generally successful with other enzymes the simplest which suggested itself, and the first therefore which we tried, was precipitation with some neutral organic solvent like alcohol, ether, or acetone. Our experiments with this method showed it to be quite useless for the case in point; but as they threw considerable light upon the properties of asparaginase they are perhaps worthy of a brief report.

(a) Precipitation of Yeast Extracts with Alcohol and Other Organic Solvents.

Ground yeast was extracted with water in the proportion of 150 cc. to every 25 gm. The activity of this extract, determined in the usual way, was such that 4 cc., acting in a total volume of 20 cc. upon an amount of asparagine containing 6.91 mg. of amide nitrogen, liberated, in 20 hours at 30°, from 47 to 50 per cent of the whole. Portions of this extract, amounting each to 200 cc., were treated with (*a*) 200 cc. of ice-cold 95 per cent ethyl alcohol, (*b*) 200 cc. of ice-cold acetone, and (*c*) a mixture of 400 cc. of alcohol and 200 cc. of ether. The resulting precipitates were separated by centrifugation or filtration, washed first with the precipitating liquid, next with cold absolute alcohol (omitted in the case of the acetone precipitate), and finally with cold anhydrous ether, dried rapidly in a current of air, and desic-

cated completely *in vacuo* over sulfuric acid. The quantity of material obtained in this way was 1.86 gm. by procedure (*a*), 2.34 gm. by (*b*), and 1.62 gm. by (*c*). Each product was now shaken up with 200 cc. of water (the volume from which it had been precipitated), and the activity of the resulting solutions or suspensions was tested under the same conditions exactly as had been that of the original extract. In no case was any appreciable quantity of ammonia liberated from the asparagine. It follows either that the enzyme had not been precipitated or that, having been precipitated, it had also been destroyed. To exclude completely the first alternative it would have been necessary to test the asparaginase activity of the filtrate from each precipitate. The bulk of the filtrate made such a direct test impracticable. It has however been shown already (see Table I) that asparaginase is almost completely destroyed by alcohol of a concentration as low as 30 per cent. In the case therefore of alcohol the second alternative is all but demonstrated to be the correct one; and it is unlikely that the effect of acetone or alcohol-ether is of a different sort.

The destructive effect of acetone accounts in all probability for the relatively low asparaginase activity of Shibata's (7) and Dox's (8) preparations of acetone-treated fungi, as well as for the entirely negative outcome of Pringsheim's (14) experiments with acetone-treated yeast.

(*b*) *Adsorption of Asparaginase by Kieselguhr and Ferric Hydroxide.*

Having failed to obtain active preparations of asparaginase by the use of alcohol or acetone, we turned next to the possibility of finding for the enzyme some suitable adsorbent. Our experiments in this direction were limited to two materials, kieselguhr and ferric hydroxide, and once again are reported more as a matter of record than for any promise they contained of a practicable method of concentration.

In the experiments with kieselguhr two 25 gm. lots of ground dry yeast were extracted in the usual way with 200 cc., and two more with 150 cc. of water. One of each pair of extracts was clarified by filtration (Preparations 13 and 15), while the other was first treated with enough kieselguhr to form a semisolid plastic mass, and was then submitted to a pressure of 400 atmos-

pheres in the Buchner press (Preparations 14 and 16). To compare the activity of these four preparations, 10 cc. of each were mixed with 10 cc. of a 1.9 per cent asparagine solution, 20 cc. of a selected 0.25 M phosphate solution, and 10 cc. of water; and for each determination (performed in duplicate) two 20 cc. portions, each of which contained 6.91 mg. of total amide nitrogen, were maintained for 20 hours at 30°. Each preparation was tested in this manner at two different levels of hydrogen ion concentration. In Table III are recorded not only the average results, but also the pH of each digesting mixture, as well as that of the phosphate solution used in making it up.

TABLE III.

Adsorption of Asparaginase by Kieselguhr.

Preparation No.	Water used to extract 25 gm.	Treatment of extract.	pH of phosphate solution used.	pH of digestion mixture.	0.02 N NH_3 found.		Amide N liberated in 20 hrs. at 30° (total = 6.91 mg.).	
					Controls.*	Main experiment.		
	cc.				*cc.*	*cc.*	*mg.*	*per cent*
13	200	Filtration.	7.62	7.18	1.78	13.38	3.25	47.0
			8.98	7.66	1.81	13.83	3.36	48.6
14	200	Kieselguhr.	7.62	7.12	2.68	5.61	0.82	11.9
			8.98	7.41	2.71	5.67	1.11	16.1
15	150	Filtration.	7.83	7.40	2.03	17.21	4.25	61.5
			9.33	7.67	2.06	17.56	4.34	62.8
16	150	Kieselguhr.	7.83	7.37	3.06	7.42	1.22	17.6
			9.33	7.61	3.03	8.10	1.42	20.5

* Including 0.16 cc. from asparagine.

The results show that kieselguhr removes from an active yeast extract a considerable fraction of its asparaginase. They confirm therefore the explanation already suggested for the relative inactivity of a yeast press juice in the preparation of which that substance has been used. At the same time, although the proportion of kieselguhr used was very large, the adsorption effected was far from quantitative. For this reason we made no further experiment in this direction with kieselguhr.

A number of experiments were next carried out with a solution of ferric hydroxide. This was prepared by shaking Merck's dialyzed iron with distilled water, until no more would readily

dissolve, and diluting the filtered product with an equal volume of water. Table IV gives the record of two representative experiments with this solution. In these a water extract of ground yeast (adjusted to have a pH in the first case of 7.0, in the second of 8.0) was diluted with varying proportions (indicated in the table) of water and ferric hydroxide. The precipitate produced by the latter, after being allowed to flocculate for 30 minutes, was separated by centrifugation. The residual activity of the supernatant liquid was then determined, in the usual way, by mixing 10 cc. with 10 cc. of a 1.5 per cent asparagine solution, 20 cc. of of buffer solution, and 10 cc. of water, and incubating a 20 cc.

TABLE IV.

Adsorption of Asparaginase by Ferric Hydroxide.

Preparation No.	Composition of preparation.					0.02 N NH_3 found.		Amide N liberated in 20 hrs. at 30° (total = 5.50 mg.).	
	Yeast extract.	Water.	Ferric hydroxide solution.	Treatment after mixing.	N in 10 cc.	Controls.	Main experiment.		
	cc.	cc.	cc.		mg.	cc.	cc.	mg.	per cent
17	50	20	0	None.	29.4	1.24	5.60	1.22	22.2
18	50	5	10	Centrifuged.	27.4	1.14	4.32	0.89	16.2
19	50	0	20	"	23.8	1.10	1.80	0.14	2.6
20	40	40	0	None.	18.3	0.94	5.12	1.17	21.3
21	40	30	10	Centrifuged.	13.3	0.89	2.67	0.49	9.0
22	40	20	20	"	10.2	0.87	1.82	0.26	4.8
23	40	10	30	"	9.2	0.84	1.15	0.09	1.6
24	40	0	40	"	7.8	0.87	1.10	0.06	1.2

portion (containing 5.50 mg. of amide N) for 20 hours at 30°. As a further indication of the effect of the ferric hydroxide, determinations were made of the total N remaining unprecipitated in a 10 cc. portion of each supernatant liquid. It will be seen from Table IV that, whenever the proportion of ferric hydroxide mixed with the yeast extract was sufficiently high (2:5 in the first experiment, 3:4 in the other), the precipitate carried down with it nearly all of the enzyme, but relatively little (one-fifth to one-half) of the total nitrogen of the extract. The enzyme could be adsorbed therefore not only quantitatively, but to a considerable

degree selectively. In this respect the use of ferric hydroxide seemed not unpromising. Unfortunately the bulk of precipitate necessary for complete adsorption was very large, and the recovery of the enzyme correspondingly difficult. It is possible that these drawbacks might have been overcome, or that an adsorbent more nearly ideal might have been discovered; but as we had meanwhile found—in precipitation of the enzyme by safranine—another procedure much more convenient and at least equally efficient, we abandoned for the present any further study of the method of adsorption.

(c) *Precipitation of Asparaginase by Safranine.*

It was observed by Robertson (16) that in solutions containing trypsin the azine color base safranine produces a precipitate; and this precipitate was afterwards shown by Holzberg (17) to be proteolytically active. Marston (18) has more recently demonstrated that safranine precipitates in a quantitative fashion not trypsin alone, but also pepsin and other proteolytic enzymes; and that, in the case at least of trypsin, 70 per cent of the active enzyme originally present can be recovered from the precipitate. Forbes (19), finally, has found that safranine may be successfully utilized for the purification and concentration of pepsin.

It appeared to us very probable that safranine would have upon asparaginase the same effect as upon pepsin or trypsin, and to test this expectation we performed first the following preliminary experiment, which was so designed as to exhibit the action of the dye at three different concentrations of hydrogen ion.

By suitable additions of 0.1 N NaOH three portions of a particular water extract of ground yeast (Preparation 25) were adjusted respectively to pH of about 6, 7, and 8, the final reaction of each being determined electrometrically. 20 cc. of each were then pipetted in duplicate into 50 cc. centrifuge tubes. To one of each pair were added 20 cc. of water, to the other 20 cc. of a 0.5 per cent aqueous solution of safranine. The dye produced in all three cases a reddish precipitate, which, after being allowed to flocculate for 30 minutes, was separated by centrifugation. The asparaginase activity of the supernatant liquid was then compared with that of its correspondingly diluted but otherwise untreated duplicate. The quantity taken for this determination

was in every case 20 cc., equivalent always to 10 cc. of the original undiluted extract; with respect to other details the procedure followed was the same as in the experiments with dialyzed iron.

TABLE V.

Effect of Safranine Precipitation upon Yeast Extracts.

In this table "supernatant" means the liquid, in volume double that of the original extract, left after removal of the safranine precipitate; while "suspension" is the precipitate itself, dispersed (within the original volume) by shaking with water.

Yeast extract.		Material tested for activity.				pH of digestion mixture.	Amide N liberated in 20 hrs. at 30° (total = 5.50 mg.).		Activity as compared with that of original extract.
Preparation No.	pH to which adjusted before precipitation.	Nature.	Amount.	Composition.					
				Solids.	N				
			cc.	mg.	mg.		mg.	per cent	per cent
25	6.07	Original extract.	10			7.91	4.76	86.6	
		Supernatant.	20			7.87	0.05	1.0	1.2
25	7.02	Original.	10			7.94	4.51	82.1	
		Supernatant.	20			7.86	0.05	1.0	1.2
25	7.98	Original.	10			8.01	4.34	78.8	
		Supernatant.	20			8.18	0.05	1.0	1.3
26	?	Original.	10			7.47	2.63	47.8	
		Supernatant.	20			7.60	0.12	2.2	4.6
		Suspension.	10			7.79	1.54	28.0	58.6
27	7.82	Original.	10		41.4	7.76	4.04	73.4	
		Supernatant.	20		33.6	7.76	0.43	7.8	10.6
		Suspension.	10		8.1	7.80	2.87	52.2	71.1
28	5.64	Original.	10	494	51.6	7.62	4.44	80.7	
		Supernatant.	20	405	42.9	7.60	0.48	8.7	10.8
		Suspension.	10	75	9.1	7.84	2.95	53.6	66.4

The results, which are to be found in Table V, show that it is possible by treatment with safranine to remove asparaginase completely, or all but completely, from solution; and further, that within the pH range of 6 to 8 the completeness of this removal is independent of the reaction.

It was to be presumed that the enzyme, removed from the extracts, would be found in the safranine precipitates. To test this presumption we determined, in further experiments, the activities not only, as before, of the original and the inactivated extracts, but also of the precipitate itself. With this object the precipitate produced by adding 100 cc. of 0.5 per cent safranine solution to 100 cc. of a yeast extract was separated by centrifugation, and transferred by means of distilled water to a 100 cc. volumetric flask. The flask was filled to the mark, and the precipitate dispersed as completely as possible by vigorous shaking. The resulting suspension was tested in aliquots of 10 cc., and its effect compared with that of 20 cc. of the supernatant liquid and 10 cc. of the original extract. The conditions under which these tests were made were the same as before.

In yet another series of experiments, similarly conducted, we included determinations, carried out upon convenient aliquots, of the total nitrogen (or of the total nitrogen and total solids) of all three of the materials of which the activities were being compared.

Representative results from these later groups of experiments are included in Table V. In attempting to give them a quantitative interpretation we have assumed what will later be shown to be within certain limits approximately true, that relative amounts of asparaginase may be estimated by the quantities of substrate decomposed in a given time. Calculations made upon this admittedly approximative basis appear in the final column of Table V, and may be taken to justifythe following conclusions:

1. While the precipitation of asparaginase by safranine is not always so complete as in the first experiment, the proportion which escapes is seldom more than 10 per cent.

2. The safranine precipitate readily hydrolyzes asparagine, and, when obtained and applied in the way described, exhibits on the average 65 per cent of the original activity of the extract, or 72 per cent of the activity which that extract has lost. Such figures should probably be taken as minimal. It was difficult to remove the safranine precipitate quantitatively from the centrifuge tubes in which it was collected; and, as it was used in the form of a suspension, which of course settled out during the digestion, it is

likely that it did not in any case have the opportunity to exert its full activity. The apparent deficit of 28 per cent does not, therefore, necessarily imply any actual destruction.

3. While the safranine precipitate contains most of the enzyme, it contains less than 20 per cent of the nitrogen and only 15 per cent of the solids of the extract. The procedure employed effects therefore a very considerable degree of purification.

Since the bulk of the precipitate produced is quite small, it can readily be suspended in a volume equal to one-fifth of that from which it has been separated. If one assumes the average yield of 65 per cent, such a concentrated suspension would possess, volume for volume, rather more than three times the activity of its original source. An enhancement of this order might be expected to provide as a rule a sufficiently active material. The following two experiments are typical of many in which this expectation has been confirmed.

1. A crude extract was prepared in the usual way by treating 375 gm. of freshly ground dry yeast with 2250 cc. of water. 2 liters of this extract were mixed with an equal volume of a 0.5 per cent aqueous solution of safranine. The precipitate, after being allowed to flocculate for 3 hours, was centrifuged off, washed once with 100 cc. of 0.5 per cent safranine, centrifuged again, and finally suspended in 250 cc. of 50 per cent glycerol. This product, which will be referred to as Safranine Concentrate II, was kept in the ice chest until required.

To determine its activity varying quantities, as shown in Table VI, were mixed with 10 cc. of a suitable phosphate solution, 5 or 10 cc. of a 1.5 per cent asparagine solution (adjusted to a pH of 7.8), and enough water to make a total volume of 25 cc. A 15 cc. portion of each such mixture was then digested for 20 hours at 30°.

2. A similar experiment was conducted with another safranine concentrate, No. IV, prepared from a different extract, and tested upon a different asparagine solution.

From the results of these experiments, as shown in Table VI, it is evident that 3 cc. of either safranine concentrate sufficed to hydrolyze completely within 20 hours the asparagine equivalent of at least 7 or 8 mg. of amide nitrogen. This corresponds with the activity which we have postulated as requisite.

The safranine method, then, enables one to produce from a crude and feeble extract a partly purified and relatively potent preparation of asparaginase. Whether it would be possible to proceed a step further, and to separate the enzyme without loss from the safranine with which it is combined, we have not determined. From such a separation, even if it were successful, there would in fact be little to be gained. Considerably more useful would be a method of converting the safranine precipitate into a dry powder, capable of retaining its activity indefinitely. Unfortunately all our attempts to prepare such a powder have been attended by an initial loss of nearly 50 per cent. We have perforce, therefore, been content to use the dye-enzyme compound always in the form of a suspension. The best medium in which to disperse it is 50 per cent glycerol, in which it settles much more slowly than in water, and in which it retains its activity longer.

TABLE VI.

Activity of Safranine Concentrates.

Per 25 cc. mixture.			Per 15 cc. fraction.		pH of digestion mixture.	Amide N liberated in 20 hrs. at 30°.	
Safranine concentrate.		Asparagine solution.	Safranine concentrate.	Total amide N.			
No.	Amount.						
	cc.	*cc.*	*cc.*	*mg.*		*mg.*	*per cent*
II	5.0	5	3.0	4.02	7.76	4.05	100.5
	0.5	10	0.3	8.04	7.96	2.28	28.4
	1.0	10	0.6	8.04	7.91	3.55	44.0
	1.5	10	0.9	8.04	7.86	4.47	55.6
	3.0	10	1.8	8.04	7.82	7.16	89.0
	5.0	10	3.0	8.04	7.74	8.09	100.5
IV	1.5	10	0.9	7.42	8.14	4.50	60.7
	2.0	10	1.2	7.42		5.47	73.7
	3.0	10	1.8	7.42		6.25	84.3
	4.0	10	2.4	7.42		6.92	93.3
	5.0	10	3.0	7.42		7.52	101.3
	6.0	10	3.6	7.42		7.52	101.3

Concentrated suspensions of the kind described have proved for many purposes both satisfactory and convenient; but they have at least one drawback likely, in certain special circumstances, to diminish their usefulness. As might be expected, they contain not

only asparaginase, but also proteases. Thus 3 cc. of Safranine Concentrate IV, which liberated in 20 hours 7.4 mg. of the amide nitrogen of asparagine, were capable of liberating also, under identical conditions, 2.5 mg. of amino nitrogen from a 2 per cent solution of Witte's peptone. Its proteolytic power was evidently small in comparison with its amidolytic; but it was not entirely negligible.

While, therefore, in studying the action of asparaginase upon pure solutions of asparagine we have been satisfied with safranine precipitation, we have continued to seek a method of concentration which should be more specific. One other method at least has shown promise of usefulness, and though we have not yet submitted it to an adequate test, we think it worthy of a preliminary mention. This method consists in precipitating the enzyme, at what is presumably its isoelectric point, by acetic acid.

(*d*) *Precipitation of Asparaginase by Acetic Acid.*

Reynolds (20) has shown that the invertase of autolyzed yeast extracts may be purified and concentrated by a process involving dialysis, removal of colloidal impurities by acidification with acetic acid, and ultrafiltration. It was in an unsuccessful attempt to apply this process to asparaginase that we discovered that enzyme to be itself precipitable by acetic acid. The conditions under which this precipitation takes place are exemplified in the following experiment.

50 cc. portions of a water extract of ground yeast, having been pipetted into six centrifuge tubes, were treated with additions of water or of glacial acetic acid, as indicated in Column 2 of Table VII. The acetic acid produced a precipitate, of which the bulk visibly increased with the quantity of acid added. After an interval of 30 minutes, to allow of flocculation, the various precipitates were separated by centrifugation. Each supernatant liquid was then submitted to determinations of total nitrogen by Kjeldahl, pH, and asparaginase activity. The five precipitates obtained were transferred with water into a single 50 cc. flask, the mixture was titrated pink to phenolphthalein, and the resulting almost clear solution was made up exactly to volume. Of this solution also we determined in a series of tests the asparaginase content. All the asparaginase determinations

were carried out by combining, in a total volume of 25 cc., 10 cc. of 1.5 per cent asparagine, 7 cc. of a 0.5 M phosphate solution of pH 8.0, and the quantities of enzyme-containing solution indicated in Column 6 of Table VII. After these mixtures, with suitable controls, had been incubated for 20 hours at 30°, the liberated ammonia was determined in portions of 15 cc. The results appear in Column 8 of Table VII.

TABLE VII.

Precipitation of Asparaginase by Acetic Acid.

Preparation No. (1)	Additions per 50 cc. of water extract. (2)	Resulting pH. (3)	N remaining unprecipitated. (4)	Material used in test. (5)	Volume used in test. (6)	pH of digestion mixture. (7)	Amide N liberated in 20 hrs. at 30° (total = 7.42 mg.). (8)	
	drops		*mg.*		*cc.*		*mg.*	*per cent*
29	10 water.	6.00	249	Original.	5	7.77	5.79	78.0
30	2 acetic acid.	5.70	252	Supernatant liquid.	5	7.82	6.01	81.0
31	5 " "	5.27	227	" "	5	8.16	3.69	49.7
32	10 " "	4.85	203	" "	5	7.95	1.25	16.9
33	15 " "	4.55	191	" "	5	7.81	0.51	6.9
34	20 " "	4.46	196	" "	5	7.82	0.28	3.8
35				Solution of combined precipitates.	1	7.96	1.63	22.0
					2	8.12	3.20	43.2
					3	8.08	5.13	69.1
					4	7.99	6.66	89.8
					5	8.12	7.50	101.0

It is evident from Table VII that the precipitates appearing at acidities between pH 5.3 and 4.5 remove increasing quantities of asparaginase from solution; that at the lowest pH attained the removal is all but complete; that the active enzyme is present in the precipitate, and can be redissolved by appropriate addition of alkali; that it is possible in this way to obtain a solution containing a higher concentration of the enzyme than the original extract; and, finally, that while acidification to pH 4.5 almost quantitatively precipitates the enzyme, it leaves most of the other nitrog-

enous constituents of the crude extract still in solution. We hope to make in the near future a further study of the possibilities of the procedure outlined.

3. Some Observations upon the Kinetics of the Asparaginase Reaction.

Having learned how to prepare a fairly active asparaginase, we endeavored to obtain with it in the first place some information concerning (1) the velocity curve of enzymatic deamidation, and (2) the relation between the speed of that reaction and the concentration of the enzyme. With this object we performed the following experiment.

Safranine Concentrate III was prepared in the same manner as No. II (see p. 213), but from a different yeast extract. Of this concentrate quantities of 15, 20, 25, and 30 cc. were pipetted serially into four 100 cc. volumetric flasks. To each flask there were added 25 cc. of a 0.5 M phosphate solution of pH 7.98, 40 cc. of a 2 per cent solution of asparagine adjusted to a pH of 8.2, and enough water to make the total volume 100 cc. From each flask nine 10 cc. aliquots were then at once measured into aeration tubes which had been placed in a water bath at 30°. The residue in each flask was used for a pH determination, the result of which varied in the four instances only from 7.91 (in that with the highest concentration of enzyme) to 7.98 (in that with the lowest). At convenient intervals between 1 and 24 hours a group of four tubes (one from each set) was removed from the water bath, and the extent to which deamidation had progressed in each was determined in the usual way. The total amount of amide nitrogen in each aliquot was calculated from the results of a Kjeldahl determination performed upon the original solution of asparagine, and was found thus to be 7.27 mg.

The results of this experiment, corrected for the usual blanks, are recorded in Table VIII, in the last four columns of which may be seen also the outcome of various attempts to find for the velocity curve of the reaction a suitable formula. Thus in Column 5 velocity coefficients have been calculated according to the linear equation $k_1 = \frac{x}{t}$. The values found indicate that the first half, or possibly, with the higher concentrations of enzyme, even more, of the velocity curve is almost a straight line.

TABLE VIII.

Rate of Deamidation of Asparagine by Varying Concentrations of Enzyme.

The total amount of amide nitrogen present in each case was 7.27 mg. This accordingly is the value given in the calculations to a.

Volume of enzyme concentrate present. (E) (1)	Time of action. (t) (2)	Amide N liberated. (x) (3)	Proportion of substrate decomposed. (4)	$k_1 = \frac{x}{t}$. (5)	$k_2 = \frac{1}{t}\log\frac{a}{a-x}$. (6)	$k_3 = \frac{1}{t}(7.3\log\frac{a}{a-x} + x)$. (7)	$k_4 = \frac{1}{t}(\sqrt{a} - \sqrt{a-x})$. (8)
cc.	*hrs.*	*mg.*	*per cent*				
1.5	2	0.69	9.5	0.35	0.022	0.50	0.070
	4	1.28	17.6	0.32	0.022	0.47	0.063
	6	1.95	26.8	0.33	0.023	0.49	0.065
	8	2.49	34.2	0.31	0.023	(0.48)	0.064
	12	3.44	47.3	0.29	0.023	0.46	0.062
	16	4.50	59.1	0.27	0.024	0.45	0.061
	20	5.34	73.4	0.27	0.029	(0.48)	0.066
	24	5.63	77.4	0.23	0.027	0.43	0.059
						Average. 0.47	
2.0	2	1.02	14.0	0.51	0.033	0.75*	0.10
	3	1.32	18.2	0.44	0.029	0.65	0.087
	4	1.73	23.8	0.43	0.030	0.65	0.087
	6	2.55	35.0	0.43	0.031	0.65	0.088
	8	3.29	45.2	0.41	0.033	0.65	0.088
	12	4.71	64.8	0.39	0.038	0.67	0.092
	16	5.56	76.5	0.35	0.039	0.63	0.087
	20	6.27	86.2	0.31	0.043	0.63	0.085
						Average. 0.65	
2.5	1	0.63	8.7	0.63	0.039	0.92*	0.120
	2	1.19	16.4	0.60	0.039	0.88	0.115
	3	1.66	22.8	0.55	0.038	0.83	0.110
	4	2.18	30.0	0.55	0.039	0.83	0.111
	6	3.13	43.0	0.52	0.041	0.82	0.111
	8	4.01	55.2	0.50	0.044	0.82	0.111
	12	5.80	79.9	0.48	0.058	0.90*	0.124
	16	6.55	90.1	0.41	0.064	0.87	0.116
	20	7.11	97.8		0.083	0.96*	0.115
						Average. 0.84	

TABLE VIII—*Concluded.*

Volume of enzyme concentrate present. (*E*) (1)	Time of action. (*t*) (2)	Amide N liberated. (*x*) (3)	Proportion of substrate decomposed. (4)	$k_1 = \frac{x}{t}$. (5)	$k_2 = \frac{1}{t}\log\frac{a}{a-x}$. (6)	$k_3 = \frac{1}{t}(7.3\log\frac{a}{a-x} + x)$. (7)	$k_4 = \frac{1}{t}(\sqrt{a} - \sqrt{a-x})$ (8)
cc.	*hrs.*	*mg.*	*per cent*				
3.0	1	0.80	11.0	0.80	0.051	1.17*	0.160
	2	1.34	18.4	0.67	0.044	0.99	0.130
	3	1.99	27.4	0.66	0.046	1.00	0.133
	4	2.51	34.6	0.63	0.046	0.96	0.130
	6	3.59	49.4	0.60	0.049	0.96	0.130
	8	4.74	65.2	0.59	0.057	1.01	0.139
	12	6.59	90.6	0.55	0.086	1.17*	0.157
	16	7.24	99.6	0.45	0.149	1.54*	0.156
	20	7.34	101.0				
						Average. 0.98	

* These figures have been omitted from the respective averages.

In a graphical representation this approximate linearity becomes, it may be said, even more evident. In cases, therefore, where the proportion of substrate decomposed is not too high (and the enzyme concentration not too low), it is possible to assume, without serious error, that equal amounts of asparagine are hydrolyzed in equal times. In the later stages of the reaction this is no longer permissible, for the coefficient k_1 ultimately begins to sink quite rapidly. On the other hand the coefficient k_2 (Column 6), calculated by the familiar equation for a monomolecular reaction, while it also remains for a time practically constant, finally rises. It might therefore be anticipated that the best description of the entire reaction would be obtained by an equation containing both a linear and a logarithmic term. An equation of this character, $k_3 = \frac{1}{t}(7.3\log\frac{a}{a-x} + x)$, has been applied to our data in Column 7. It possesses, as may be noted, the same general form, $kt = m\log\frac{a}{a-x} + nx$, as that found by Michaelis and Menten (21) to describe the hydrolysis of sucrose by invertase. In the equation

of Michaelis and Menten m and n are complex constants derived from the independently measured affinity constants of the enzyme for its substrate and its products; and the formula, as they use it, expresses rationally the influence upon the reaction rate of the several enzyme combinations formed. In applying it to our own data we have taken n arbitrarily as 1, and have found the appropriate value of m by calculation from one pair of experimental data—those, namely, obtained at 8 and 20 hours for $E = 1.5$. The number 7.3 is therefore here a purely empirical constant, and we have at present no evidence to show that it could be interpreted in the sense required by the theory of Michaelis. All that can be asserted is that its use does furnish an equation which fits the results, on the whole, exceedingly well. Conspicuous departures from constancy of k_3 appear with regularity only towards the end of the reaction. Occasional discrepancies at the beginning may be due to analytical errors involved in the measurement of small values of x. These apart, the equation may be said to describe quite exactly from four-fifths to nine-tenths of the entire course of the reaction.

If the average velocity constants (k_3), as calculated in Column 7 of Table VIII, are divided by the relative concentrations (E) of the enzyme, as given in Column 1, the values obtained (0.313, 0.325, 0.334, and 0.327) are practically identical. This means that, within the range represented, the velocity of the reaction is proportional to the concentration of enzyme. Under those conditions which permit the assumption that $x = kt$ it follows, then, that relative concentrations of enzyme may be measured by the amounts of substrate decomposed in equal times. The data of Table VIII will be found to supply experimental justification of this deduction.

In several respects the results just discussed are confirmatory of conclusions already reached by Clementi and Cantamessa (22). These authors found for instance, like us, that velocity coefficients calculated according to the monomolecular law usually rise as the action of asparaginase proceeds; and in some instances at least they found a practically linear relation between the quantity of asparagine hydrolyzed and the time. In their experience the nearest approach to a constant coefficient of velocity was given by the equation of Abderhalden and Fodor (23), $kt = \sqrt{a} - \sqrt{a - x}$, an equation which implies that the speed of the enzyme

reaction is controlled by a phenomenon of adsorption. Column 8 of Table VIII shows that this equation fits our data just as well as the one we have ourselves made use of; but, since it fits them no better, this can hardly be taken as proof that adsorption rather than chemical affinity is the force determining the action of the enzyme.

As a matter of fact, we have observed that neither the one equation nor the other will fit the course of the asparaginase reaction under all conditions. The form of the velocity curve is probably to a large extent dependent upon the relative concentrations of enzyme and substrate. The conclusions drawn from Table VIII apply in strictness only to the comparatively narrow range of conditions covered by the experiment described. The conditions there chosen were those under which we expected to put the enzyme to practical use; and it would have led us too far from our immediate object to study in detail the influence of wider variations.

4. Relation of Hydrogen Ion Concentration to the Activity of Asparaginase.

Euler (24), in experiments of which the details have not been published, found that the "desamidase" of yeast acted most energetically upon asparagine in the region of pH between 7.6 and 8.0. Clementi (4) stated that his asparaginase was most active at neutral or faintly alkaline reactions, and that it was inhibited by small concentrations of free acid. Since it was evidently desirable to possess more detailed information concerning this aspect of the enzyme's behavior, we have conducted quite a number of experiments both with crude extracts of yeast and with safranine concentrates. All gave essentially the same results, so that it may suffice to report only a selected number of those in which a safranine preparation was employed.

The experiments of which the results are shown in Table IX were performed as follows: An enzyme solution of suitable concentration was prepared by diluting 115 cc. of Safranine Concentrate II with 135 cc. of 50 per cent glycerol; by combining KH_2PO_4 and NaOH in varying proportions, 0.25 M phosphate mixtures were obtained having a pH ranging from 5.6 to 10.7; while to provide the substrate a 2 per cent solution of asparagine was ad-

justed with 0.1 N NaOH to a pH of 7.8. Mixtures were then made, each of which contained 10 cc. of the asparagine solution, 10 cc. of a phosphate mixture, and 4 cc. of the enzyme suspension. To some of these mixtures, as indicated in Table IX, there were added, in order to reach the higher degrees of alkalinity, small,

TABLE IX.

Effect of Varying Hydrogen Ion Concentration upon Activity of Asparaginase.

pH of phosphate mixture used.	Normal NaOH added to digest.	pH of complete digestion mixture.	Amide N liberated in 20 hrs. at 30° (total = 11.11 mg.).		Proportion of total enzyme active.
	drops		*mg.*	*per cent*	*per cent*
5.50		5.82	0.32	2.9	6.2
5.66		5.93	0.49	4.4	9.5
5.95		6.13	0.45	4.1	8.7
6.15		6.30	0.76	6.9	14.7
6.20		6.47	1.57	14.2	30.4
6.54		6.70	2.36	21.3	45.6
6.74		6.89	3.10	28.0	60.0
7.02		7.11	3.86	34.7	74.6
7.22		7.36	4.38	39.6	84.7
7.44		7.52	4.53	40.8	87.6
7.98		7.90	5.17	46.7	100.0
7.98		7.87	5.15	46.5	99.6
9.04		8.14	5.07	45.8	98.1
10.30		8.56	4.70	42.5	90.9
8.75	10	8.89	4.28	38.7	82.8
9.04	12	9.02	3.96	35.8	76.6
10.38	12	9.15	3.70	33.4	71.6
10.68	3	9.33	3.49	31.5	67.5
10.68	6	9.50–9.45	3.05	27.6	59.0
10.10	16	9.79–9.69	1.71	15.4	33.1
10.68	9	9.87–9.74	2.27	20.4	43.9
10.68	12	10.18–9.83	0.81	7.3	15.7
10.68	14	10.21–9.99	0.39	3.5	7.5
10.68	17	10.41–10.01	0.00	0.0	0.0

roughly measured amounts of normal NaOH. All were then made up to a volume of 25 cc., and set in a water bath at 30°. A few cc. in each case were at once withdrawn for the determination of the initial pH. At the end of 20 hours 15 cc. were taken for the estimation of the liberated ammonia. The residue was used for a second measurement of pH at the end of the digestion.

An appreciable difference between initial and final pH was observed, and is recorded, only in the most alkaline mixtures, of which the buffer value was practically *nil*.

It was necessary, for practical reasons, to divide the entire experiment into two parts, of which the second was performed after the first had been completed. The first dealt with the pH range from 5.8 to 7.9; the second with 7.9 to 10.4. The final mixture of the first set was repeated as the first mixture of the second. The fact that both gave the same result shows that the two sets may be regarded as forming a single series.

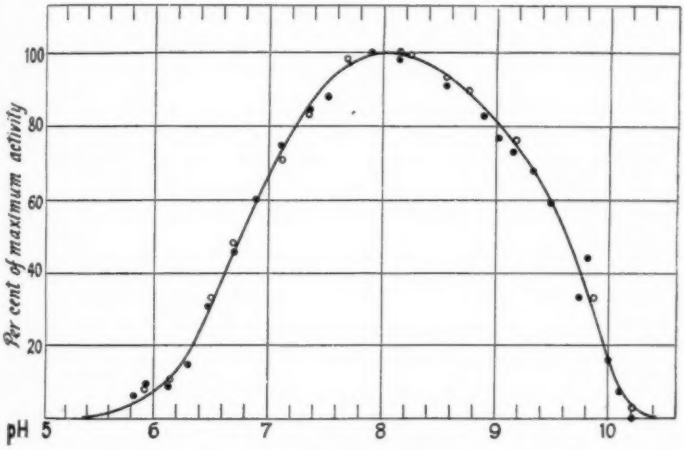

CHART 1. Relation of activity of asparaginase to pH. The solid circles represent points taken from Table IX; the clear circles, points yielded by another experiment.

From a Kjeldahl analysis of the asparagine solution it was ascertained that the total amount of amide nitrogen in each 15 cc. sample was 11.11 mg. From the results in Table IX it will be observed that the amount of enzyme was so chosen that at most rather less than half of this total was liberated within the time allowed. Under these circumstances, it has already been shown, the amount of substrate decomposed in a given time is almost directly proportional to the concentration of enzyme. At each

pH therefore the relative amount of enzyme active may be taken as measured by the amount of amide nitrogen which it liberated in the experiment. It is upon this basis, and with the amount of enzyme active at the optimum taken as 100, that the figures in the last column of Table IX have been calculated.

A graphical representation of these figures will be found in Chart 1, upon which have been plotted, for further illustration, the results of a second experiment conducted under the same conditions. In the construction of this graph we have used, at the highest alkalinities, that value of pH which lies midway between the initial and the final ones observed. The smooth curve drawn through the experimental points would indicate that the optimum lies between pH 7.9 and 8.1, which is in fair agreement with the statement of Euler. That the curve is not absolutely symmetrical about this optimum may or may not be the result of experimental errors or of the method of calculation. With respect to the ranges covered respectively by the ascending and the descending limb, symmetry is almost exact. The total range within which the enzyme exerts a detectable activity extends apparently from pH 5.5 to 10.3.

Further experiments, in which the conditions (especially the concentration of enzyme) were somewhat varied, have given curves entirely similar in form to the one presented, but occupying sometimes a slightly different position upon the scale of pH. In the most extreme instance of such a displacement all of the points lay about 0.25 pH units to the left, so that the optimum, for instance, instead of being at 8.0, was at 7.75. The cause of this variability, which is not without its precedent, we have not sought to discover.

5. Inactivation of Asparaginase by Heat.

Our experience in handling asparaginase has taught us that it is in many ways exceedingly liable to destruction or inactivation; and one factor of which we have specifically studied the deleterious influence is heat. The rapid disappearance of the enzyme even at but moderately elevated temperatures is illustrated by the following experiment.

30 cc. of Safranine Concentrate II were diluted with 50 per cent glycerol to 100 cc.; and 4 cc. of this diluted asparaginase were

mixed, in each of sixteen tubes, with 5 cc. of a phosphate buffer solution of pH 8.18. The mixtures, which had a pH of 7.87, were maintained, in pairs, for 20 hours at the different temperatures noted in Table X. At the end of this exposure they were cooled or warmed to 30°, and to each were added 5 cc. of 2 per cent asparagine. The ammonia subsequently liberated in each tube was determined after the usual interval of 20 hours. The results, as recorded in Table X, show that even at a temperature of 30° more than half of the enzyme had disappeared within 20 hours; at 50° its residual activity was then barely perceptible; while at 55° its destruction was already complete. These effects occurred at a pH which is optimum for the activity of the enzyme, and in the absence of the substrate. How far they would be modified

TABLE X.

Heat Inactivation of Asparaginase.

Temperature to which enzyme was previously exposed.	Time of exposure.	Temperature and time of subsequent digestion.	Amide N liberated (total = 9.26 mg.).	
°C.	hrs.		mg.	per cent
3	20	20 hrs. at 30°.	4.16	44.9
30	20	20 " " 30°.	1.74	18.8
35	20	20 " " 30°.	0.92	9.9
40	20	20 " " 30°.	0.65	7.0
45	20	20 " " 30°.	0.10	1.1
50	20	20 " " 30°.	0.07	0.7
55	20	20 " " 30°.	0.00	0.0

by variations in hydrogen ion concentration or by the presence of asparagine, we cannot at present say; neither can we tell to what extent they may have been due not to heat *per se*, but to the enhanced activity of some accompanying protease.

In an earlier experiment, recorded in Table I, we observed in a crude yeast extract a rate of inactivation at 35° much inferior to that now found in a safranine concentrate. The difference suggests the presence in the cruder material of some substance or substances that have a protective action.

The experiment of Table X shows that safranine concentrates, if they are to retain their activity for any length of time, should be preserved at a temperature as close as possible to the freezing point.

6. Specificity of Asparaginase.

In the experiments already described there may be found sufficient evidence that yeast asparaginase removes by hydrolysis only the amide group of its substrate, leaving the amino group entirely unaffected. Thus in Table VI it is shown that neither by increasing the relative amount of enzyme nor by diminishing the relative amount of substrate is it possible to obtain from asparagine more than half of its total nitrogen; while from Table VIII (with $E = 3.0$) it appears that once the amide nitrogen has been completely liberated (at 16 hours) a prolongation of the action (to 20 hours) adds no appreciable quantity to the ammonia obtained. The absence of a deaminizing action in our preparations hardly required further demonstration; but we have clinched the point by conducting several experiments in which solutions of sodium aspartate were incubated for 48 hours with the active Safranine Concentrate II. In no case did we obtain any significant amount of ammonia. The enzyme is specific therefore in the sense that it can hydrolyze only an amide group.

The records of the literature leave it doubtful whether the enzyme hydrolyzing asparagine can attack in a similar manner other amides. We have therefore tested the action of our Safranine Concentrate II upon urea, formamide, acetamide, propionamide, valerianamide, succinamide, oxamide, and salicylamide. The first five of these were used in the form of 1 per cent aqueous solutions, succinamide as a saturated solution (0.45 per cent at 15°), and the remaining two (which are nearly insoluble) as 1 per cent suspensions of the finely ground material. In each test 5 cc. of the amide solution (or suspension) were mixed, in duplicate, with 10 cc. of phosphate buffer (pH 8.18) and 3 cc. of the enzyme suspension; the volume was made up to 25 cc.; and the ammonia of a 15 cc. fraction was determined after 20 hours of digestion at 30°. Suitable blanks were run in each case, and for comparison a similar experiment was run simultaneously in which the substrate was 10 cc. of 1.5 per cent asparagine. The asparagine mixture yielded 7.17 mg. of ammonia nitrogen. None of the others yielded any more ammonia than the corresponding blank. It is certain therefore that our preparation had no action whatever upon any of the amides tested.

Some time after the joint work here under report was completed, one of us (A. H.) received from Dr. H. B. Vickery of the Connecticut Agricultural Experiment Station (to whom grateful acknowledgment is now made) a small specimen of glutamine. A few preliminary experiments conducted in this laboratory by Mr. H. B. Collier have shown that this biologically important homologue of asparagine undergoes in feebly alkaline phosphate mixtures at 30° a slow spontaneous hydrolysis; but that in the presence of our yeast enzyme this hydrolysis is greatly accelerated. It appeared, though, that under like conditions the enzymatic hydrolysis of glutamine is rather less rapid than that of asparagine. Further experiments with glutamine will be undertaken as soon as a further supply of the material is available.

7. *Asparaginase in Calf Liver.*

While yeast proved to be in our hands an entirely convenient source of asparaginase, we have not neglected to test the possibility of obtaining it also from an animal source. With this object we prepared from the liver of the calf (1) an extract obtained by treating the finely ground organ with an equal weight of 50 per cent glycerol, (2) a precipitate produced by adding to some of this extract 2 volumes of ethyl alcohol mixed with 1 volume of ether, (3) a dry liver powder made according to the directions of Wiechowski (25). In parallel experiments we then tested the effect, upon 10 cc. of 1.5 per cent asparagine solution, of (*a*) 5 cc. of the glycerol extract, (*b*) 0.2 gm. of the dried alcohol-ether precipitate, and (*c*) 0.5 gm. of the whole liver powder. In each case the mixture was treated with 10 cc. of a suitable buffer solution and enough water to make a total volume of 25 cc.; the ammonia was determined, after 20 hours at 30°, in 15 cc. The test with the glycerol extract yielded 2.68 mg. of ammonia nitrogen, which was 36 per cent of the total amide nitrogen available (7.42 mg.). The other two preparations showed no activity whatever. It appeared therefore that the liver, as Lang and Clementi had claimed, contains asparaginase; and that this liver enzyme, like that of yeast, is very labile, being destroyed by drying or by contact with alcohol and ether. Clementi (4), it is true, found that the alcohol and acetone precipitates, which he prepared from guinea pig liver, were by no means inactive; but his results do not allow us to compare their potency with that of the original extracts.

The activity of our liver extract was inferior to that of similarly

prepared extracts of yeast; but it would be unfair to generalize a single observation of this kind.

SUMMARY.

1. The enzyme asparaginase may be obtained by water or glycerol extraction of thoroughly disintegrated yeast cells.

2. It is present also in the liver of the calf.

3. It is very labile, being rapidly destroyed not only by heat but also by contact with cold alcohol or acetone.

4. It is adsorbed by kieselguhr and by ferric hydroxide.

5. It may be precipitated by adjusting the pH of its solution to approximately 4.5.

6. It may be conveniently concentrated and partly purified by precipitation with safranine; but the product thus produced is still contaminated by protease.

7. The active enzyme obtained by the safranine method removes quantitatively as ammonia the amide nitrogen of asparagine, but leaves the amino group intact.

8. Of a number of other acid amides tested the enzyme was found to hydrolyze only glutamine. Its action is therefore limited, as far as known, to the amides of the two amino acids, aspartic and glutamic.

9. The course of the enzymatic hydrolysis of asparagine is not described by the law applicable to a simple monomolecular reaction; coefficients of velocity calculated by that law increase as the reaction proceeds.

10. Nearly constant coefficients are yielded by the equations $kt = m \log \frac{a}{a - x} + x$ (in which m is an empirical constant) and $kt = \sqrt{a} - \sqrt{a - x}$.

11. Asparaginase exerts its maximum activity at pH 7.9 to 8.1, and its range extends on either side of this optimum to about 5.5 and 10.3.

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A NEW DIFFERENTIATION BETWEEN THE ANTINEURITIC VITAMIN B AND THE PURELY GROWTH-PROMOTING VITAMIN B.*

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It has become increasingly clear that vitamin B consists of at least two substances, but that both of these occur in some of the well known sources of vitamin B, *e.g.* yeast. The most important early evidence for this separation of vitamin B rested upon small quantitative differences in the distribution of the two substances in the foods containing them. Recently differences in stability and solubility of these substances have added their weight to the belief that there is an antineuritic and a purely growth-promoting water-soluble vitamin.

A comprehensive review of the literature will not be given in this paper. The recent article of Chick and Roscoe (1) refers to most of the recent articles on this subject. The short paper of Smith and Hendrick (2) gave excellent evidence of the presence of two factors in yeast, the antineuritic vitamin B and a thermostable growth-promoting vitamin. 2 weeks later, the longer and more comprehensive paper of Goldberger, Wheeler, Lillie, and Rogers (3) was published. This work has left no doubt as to the presence of two factors in yeast, the factor P-P (as yet not separated from the growth-promoting vitamin B) and the antineuritic vitamin. These authors also point out that maize is a rich source of the antineuritic vitamin but is very low in the thermostable growth-promoting factor.

Hauge and Carrick (4) soon after published work done on chickens, which agreed in every respect with that of Goldberger *et al.* They had obtained a dried brewers' yeast which was very low in the antineuritic vitamin but quite rich in the growth-promoting factor. As a consequence the birds which grew normally soon came down with polyneuritis. They found corn and corn germ to be rich sources of the antineuritic vitamin, yet very low in the other growth-promoting factor. The recent papers of Hassan and

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XUM

Drummond (5) and of Chick and Roscoe (1) have confirmed the earlier work by showing that yeast contains a thermolabile antineuritic vitamin and a thermostable growth-promoting vitamin. The latter authors have added the observation that wheat germ is relatively poor in the growth-promoting vitamin B. Salmon (6) has also shown an unequal distribution of the two factors in the seeds and leaves of the soy bean and velvet bean. He has also found that an extract adsorbed on fullers' earth was rich in the antineuritic vitamin, while the residue carried all of the other growth-promoting factors.

In working with the highly purified diet, No. 519 (Table I), this laboratory has come upon an almost perfect separation of the

TABLE I.

Ingredients.	Diet 232.	Diet 387.	Diet 519.	Diet 520.	Diet 523.
Casein (commercial)	32.0	30.0		25.8	30.0
" (highly purified)			24.0		
Salts (185)*	4.0	4.0	3.8	3.8	4.5
Corn-starch (cooked)	40.0	46.0			
Lard (commercial)	22.0	20.0			20.0
Sucrose (commercial)				70.4	45.5
" (recrystallized)			72.2		
Cod liver oil (Patch)	2.0	†	†	†	†

* McCollum, E. V., and Simmonds, N., *J. Biol. Chem.*, 1918, xxxiii, 63.
† 3 drops fed daily.

antineuritic and growth-promoting vitamins B. It will be noted that Diet 232 is made of ordinary commercial products while Diet 519 contains only Van Slyke casein, recrystallized sucrose, and highest purity salts. When Diet 519 is supplemented by dried yeast (0.7 gm. daily) and cod liver oil (3 drops daily), good growth ensues for a while and the curves assume a plateau when the rats are about 130 to 150 gm. in weight (Fig. 1). If lard devoid of vitamin B be added to this diet, the growth is normal. It is apparent, therefore, that our yeast contains both of the vitamin B factors,[1] for the failure to grow to normal weight on

[1] The experiments reported in this paper have all been conducted with a single source of whole dried yeast, that kindly furnished by the Fleischmann Company of New York, and presumably their strain F. We have recently found indications of considerable variation in the amount of growth-promoting vitamin B in yeasts; in fact, 700 mg. daily of one sample tested by us gave growth and ovulation superior to that secured with 1 gm. daily of the yeast here employed.

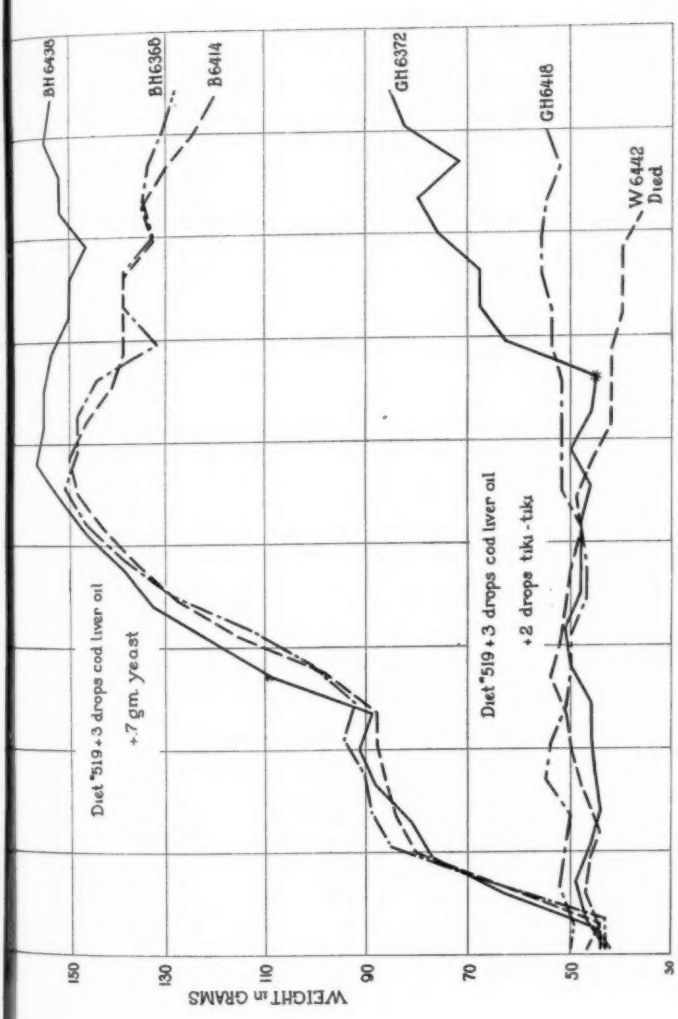

FIG. 1. Individual growth curves showing the very inferior growth of animals on Diet 519 + 2 drops of tiki-tiki when compared with litter mates on Diet 519 + 0.7 gm. of yeast. The asterisk (*) on the curve of Rat GH6372 indicates change in diet, 0.7 gm. of yeast added.

TABLE II.

Showing Value of Tikitiki as Supplement to Diets 232 and 519.

Diet	Rat No.	Age at rupture of vaginal membrane.	Age at first estrus.	Ovulation cycles (length in days).	Weight at 60 days.	Weight at 90 days.	Weight at 120 days.
		days	*days*		*gm.*	*gm.*	*gm.*
Diet 519 + 2 drops tikitiki, 3 drops cod liver oil.	GH6372				46	48	50
	GH6418				54	47	52
	W6442				50	51	42
Diet 519 + 6 drops tikitiki, 3 drops cod liver oil.	GH6373	122		No cycles.	66	71	79
	BH6419	69	72	12	60	71	80
	E6443	74		No cycles.	62	59	52
Diet 232 + 3 drops tikitiki.	B7312	42	45	4, 5, 6, 11, 3, 6, 7, 34+	136	140	130
	BH7293	41	43	5, 4, 6, 6, 7, 4, 5, 4, 4, 24, 11	156	184	182
	W7286	41	44	7, 12, 5, 6, 5, 5, 6, 34+	137	150	158
Diet 519 + 3 drops tikitiki, 3 drops cod liver oil.	B7313	Not open at 120 days.			60	66	66
	W7292	46	56	12, 10, 9, 35+	59	70	72
	W7287	117		No cycles.	56	72	70
Diet 519 + 0.7 gm. yeast, 3 drops cod liver oil.	BH6368	66	87	5, 6, 5, 4, 5, 5, 5, 5, 53+	94	128	148
	E6414	81	81	5, 6, 5, 6, 6, 5, 7, 9, 6, 6, 46+	88	127	146
	BH6438	79	89	7, 8, 24, 13, 16, 29+	91	132	156

+ indicates that the last recorded period was not terminated by estrus at the time the data were tabulated.

XUM

Diet 519 is due to a new deficiency remedied by lard, lettuce, and beef liver (7).

Now if Diet 519 is supplemented by 2 drops of the antineuritic

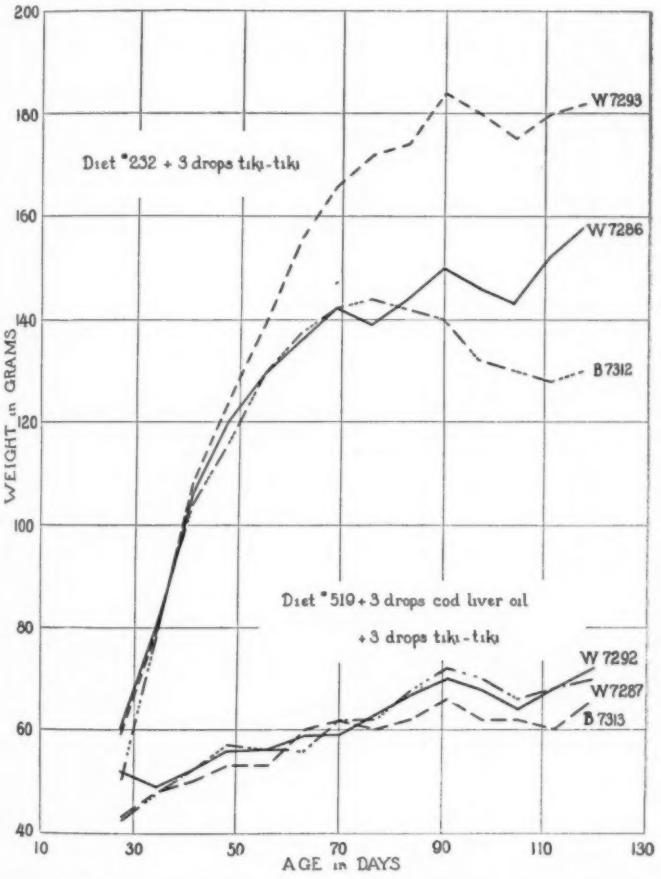

FIG. 2. Individual growth curves showing the inferior growth of animals on Diet 519 + 3 drops of tikitiki when compared with litter mate sisters on Diet 232 + 3 drops of tikitiki.

XUM

TABLE III.

Showing Distribution of Growth-Promoting Vitamin B in the Components of Diet 232.

Diet.	Rat No.	Age at rupture of vaginal membrane.	Age at first estrus.	Ovulation cycles (length in days).	Weight at 60 days.	Weight at 90 days.	Weight at 120 days.
		days	*days*		*gm.*	*gm.*	*gm.*
Diet 519 + 3 drops tikitiki, 3 drops cod liver oil.	BH7261	73		No cycles.	78	68	65
	BH7253	39	39	7, 5, 12, 6, 8, 47+	80	84	80
	G7211	75	84	41	70	90	88
	GH7213	Not open at 104 days.			46	48	Died at 104 days.
	B7245			No cycles.	78	100	90
	BH7207	63	72	18, 34+	89	98	91
Diet 520 + 3 drops tikitiki, 3 drops cod liver oil.	BH7260	59	59	6, 17, 40+	89	104	110
	G7212	40	44	15, 8, 63+	109	118	120
	B7256	74	81	42+	56	108	116
	BH7214	71		No cycles.	98	108	100
	B7244	63	63	7, 53+	120	131	136
	G7206	42	42	9, 21, 7, 32+	101	116	120
Diet 525 + 3 drops tikitiki, 3 drops cod liver oil.	BH7259	58	58		80	Died at 75 days.	
	B7247	74	74	48+	60	112	130
	BH7243	70		No cycles.	90	140	156
	GH7255	61	61	7, 6, 13, 25, 11	80	130	148
	B7205	49	60	11, 5, 5, 6, 37+	72	100	110

	BH7243	70		No cycles.	90	140	156
	GH7255	61	61	7, 6, 13, 25, 11	80	130	148
	B7205	49	60	11, 5, 5, 6, 37+	72	100	110
Diet 387 + 3 drops tikitiki, 3 drops cod liver oil.	BH7258	37	37	5, 6, 5, 7, 6, 11, 48+	128	124	136
	G7246	60	60	9, 9, 4, 41+	131	144	158
	GH7254	32	32	7, 7, 5, 5, 5, 8, 5, 7, 5, 5, 5, 6, 5, 5, 5, 5	142	178	178
	BH7248	45	45	5, 5, 5, 11, 7, 4, 4, 5, 4, 6, 4, 6, 12+	124	161	170
	B7204	50	50	8, 6, 25, 34+	131	124	136
	B7203	41	41	6, 6, 5, 5, 5, 3, 5, 4, 4, 4, 4	144	180	194
Diet 520 + 0.7 gm. yeast, 3 drops cod liver oil.	W7200	48	50	9, 27, 37+	126	122	112
	W7218	55	55	67+	149	150	156
	B7176	41	44	11, 8, 48, 13+	130	132	136
	W7185	46	46	14, 12, 16, 36+	120	140	146
	BH7190	53	53	71+	130	130	139
	B7193	59	62	32, 12, 18+	120	124	128

+ indicates that the last recorded period was not terminated by estrus at the time the data were tabulated.

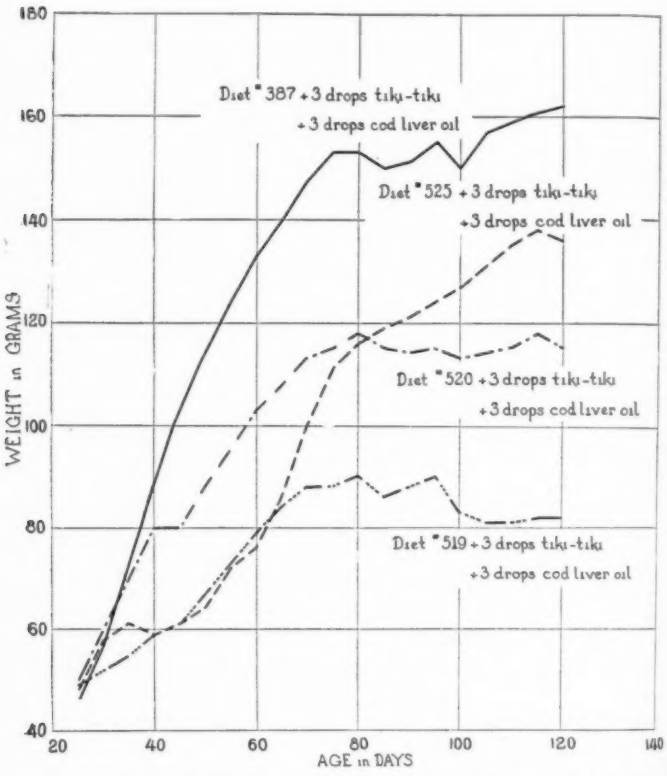

FIG. 3. Composite growth curves (six animals each, litter mates) showing the better growth of animals when commercial casein and commercial cornstarch are used in the diet.

concentrate tikitiki,[2] only the slightest growth can take place, the animals hardly increasing their weaning weights, yet without suffering from any evident upset over a period of from 4 to 6 months. Even when the dose is trebled (6 drops daily) the

[2] *Philippine J. Sc.*, 1921, xix, 67. Tikitiki is the dilute alcoholic extract of white rice polishings made by the Philippine Bureau of Science for distribution to the natives of the islands. We are greatly indebted to Dr. A. H. Wells, chemist in charge, for our supply of this valuable concentrate of antineuritic vitamin B.

animals grow insignificantly, attaining about 85 gm. at the close of a half year (Fig. 1). On the other hand, the more impure diet, No. 232, without yeast, shows very considerable growth when supplemented by 2 or 3 drops of tikitiki daily (Table II and Fig. 2). The results show clearly that tikitiki is almost entirely lacking in growth-promoting vitamin B though possessing the antineuritic vitamin. When both yeast and tikitiki are withheld from rats on Diet 519, the animals cease growing at once. Death is invariable within 3 weeks.

Sources of the Growth-Promoting Vitamin B in Diet 232.

It is evident from Table II and Fig. 2 that Diet 232 contains enough growth-promoting vitamin B for very considerable growth when 3 drops of tikitiki are fed daily to furnish the antineuritic vitamin. It became of interest then to find just what ingredient of the diet carried most of the growth-promoting factor. The results of these experiments are given in Table III and Fig. 3.

The somewhat improved performance of animals on Diet 519 in this group can be referred to the facts that new tikitiki was used and that the animals were kept in larger cages with three in a cage rather than in individual cages. The survey of Fig. 3 shows a very considerable growth-promoting vitamin B carried in the commercial casein and commercial sugar when compared with the pure ingredients (Diet 520 compared with Diet 519). When lard is added to the casein and sugar (Diet 525), a still further acceleration of growth results. We have already stated that we refer this to the presence in lard of what is probably a new growth-promoting factor. If Diet 525 is changed only by the substitution of corn-starch for sugar (Diet 387), further acceleration of growth results—an acceleration which we believe can be referred to the increased stores of growth-promoting vitamin B in corn-starch when compared with sugar.

CONCLUSIONS.

1. A new and striking differentiation has been secured between the antineuritic vitamin B and the purely growth-promoting vitamin B.

2. The concentrate of antineuritic vitamin B, called tikitiki, is almost lacking in growth-promoting vitamin B.

3. Growth-promoting vitamin B is carried both by commercial corn-starch and by commercial casein.

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STUDIES ON THE OXYGEN-, ACID-, AND BASE-COMBINING PROPERTIES OF BLOOD.

III. THE VALIDITY OF HYDROGEN ION ACTIVITY DETERMINATIONS BY THE HYDROGEN ELECTRODE IN SYSTEMS CONTAINING CARBONIC ACID, CARBONATES, HEMOGLOBIN, CARBON MONOXIDE HEMOGLOBIN, AND METHEMOGLOBIN.

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[1] A table of the symbols used in this paper will be found at the end of Paper V.

INTRODUCTION.

The theory of the hydrogen electrode is so well known that we give here no more than will make the purpose of this paper clear.

If

a_{H^+} = known hydrogen activity of some convenient reference solution, *e.g.* 0.1 N HCl

a'_{H^+} = unknown hydrogen activity of any solution

P_{H_2}, P'_{H_2} = the respective hydrogen pressures with which the solutions are in equilibrium

then if each solution is set up as a half-cell in equilibrium with gaseous hydrogen at the respective partial pressures P_{H_2}, P'_{H_2} the combination forms the cell

$$\text{Pt} \mid \text{H}_{2(P_{H_2})} \mid 0.1\ \text{N HCl}\ (a_{H^+}) \parallel (a'_{H^+}) \mid \text{H}_{2(P'_{H_2})} \mid \text{Pt}$$

Liquid junction

Neglecting the liquid junction for the moment, we find the electromotive force of this cell is

$$E = \frac{RT}{N\mathbf{F}} \ln \frac{a_{H^+}}{a'_{H^+}} \left(\frac{P'_{H_2}}{P_{H_2}}\right)^{\frac{1}{2}} \tag{1}$$

where R = gas constants in electrical units = 8.316 joules per degree
T = absolute temperature
N = No. of gm.-ions = 1
$\mathbf{F}$ = 1 faraday = 96,494 coulombs per equivalent

Substituting into Equation 1 these values and transposing to Briggsian logarithms, we get

$$E = 0.0001984\ T \log \frac{a_{H^+}}{a'_{H^+}} \left(\frac{P'_{H_2}}{P_{H_2}}\right)^{\frac{1}{2}} \tag{2}$$

In practice each half-cell is set up with a stable reference half-cell of constant E.M.F. = e_0 giving the cells

$$\text{Pt} \mid \text{H}_{2(P_{H_2})} \mid 0.1\ \text{N HCl}_{(a_{H^+})} \parallel \text{Reference cell}_{(e_0)},\ \text{E.M.F.} = E_1$$

L_1

$$\text{Pt} \mid \text{H}_{2(P'_{H_2})} \mid (a'_{H^+}) \parallel \text{Reference cell}_{(e_0)},\ \text{E.M.F.} = E_2$$

L_2

Again, if we disregard the liquid junction potentials L_1 and L_2, it is clear that $E = E_1 - E_2$.

From these observed E.M.F. determinations a'_{H^+} may be calculated by Equation 2, provided we make certain assumptions. Some of these assumptions are known to be inexact. The limitations which these assumptions put on the electrometric determination of a_{H^+} (or pa_H, the negative logarithm of this value) have on the whole been clearly realized for simple aqueous solutions. However, in biological fluids containing protein, $BHCO_3$, H_2CO_3, CO_2, and BCl, the amount of investigation designed to test them has been limited.

In this paper we have outlined these basic assumptions and have presented experimental evidence bearing on them wherever possible. Although we have used hemoglobin $NaHCO_3$, H_2CO_3, CO_2, NaCl systems exclusively, our experimental results are, we believe, of significance in the general subject of the validity of pa_H determinations in protein-containing solutions.

Assumptions Which Must Be Made to Calculate pa_H in $BHCO_3$-H_2CO_3-Hb Systems.—These assumptions may be conveniently given under the following captions but will not be discussed in strict order.

I. Assumptions which affect the absolute magnitude of the pa_H of the unknown solution.
   1. pa_H of the standard reference solution.
   2. Liquid junction potential of reference solution, *e.g.* 0.1 N HCl and reference half-cell.
   3. Reproducibility and constancy of the hydrogen electrode.
   4. Absence of appreciable side reactions.
   5. Hydration of the hydrogen ion in hemoglobin solution.

II. Assumptions which affect the relative magnitude of the pa_H of the unknown solution.
   A. At constant temperature.
   6. Liquid junction potential of unknown solution and reference half-cell.
   7. Reversibility of the E.M.F. of hydrogen electrode in Hb-$NaHCO_3$, H_2CO_3, NaCl solutions.
   B. At varying temperature.
   8. Temperature effect on pa_H of reference solution.
   9. Temperature effect on liquid junction potentials of the unknown solution, the reference solution, and reference half-cell.

a_{H^+} of Reference Solution and Liquid Junction Potential of Reference Solution and Reference Cell.—The prevailing cell system for pa_H determinations and the one used throughout the work here is composed of the two cells

1. $\mathrm{Hg} \left| \begin{matrix} \mathrm{HgCl} \\ \text{in saturated} \\ \mathrm{KCl} \end{matrix} \right| \begin{matrix} \text{Saturated} \\ \mathrm{KCl} \end{matrix} \Big\| 0.1\ \text{N HCl}_{(a_{H^+})} \Big| \mathrm{H_2}_{(P'_{H_2})} \Big| \mathrm{Pt} \Big| \ldots . E_1$

L_1

2. $\mathrm{Hg} \left| \begin{matrix} \mathrm{HgCl} \\ \text{in saturated} \\ \mathrm{KCl} \end{matrix} \right| \begin{matrix} \text{Saturated} \\ \mathrm{KCl} \end{matrix} \Big\| \begin{pmatrix} \mathrm{NaHCO_3} \\ \mathrm{NaCl} \\ \mathrm{Hb} \end{pmatrix}_{a'_{H^+}} \Big| \mathrm{H_2}_{(P'_{H_2})} \Big| \mathrm{Pt} \Big| \ldots . E_2$

L_2

The saturated calomel half-cell is merely a convenient and constant source of E.M.F. whose E.M.F. value, e_0, is calculated by the equation

$$e_0 = E_1 - 0.0001984\ T \log a_{H^+} \left(\frac{760}{P_{H_2}}\right)^{\frac{1}{2}} - L_1 \qquad (3)$$

from the values of activity of the hydrogen ion in 0.1 N HCl and the liquid junction potential L_1. But neither a_{H^+} nor L_1 is known. As an approximation we assume the liquid junction potential to be zero and take $10^{-1.08}$ (from 15–40°) as the a_{H^+} of 0.1 N HCl. But this value is the *mean ion activity product.* $a_{HCl} = \sqrt{a_{H^+} a_{Cl^-}}$ of HCl as determined by Noyes and Ellis (1917) in cells without liquid junction potential. In other words we assume $a_{H^+} = a_{Cl^-}$; *i.e.*,

$$a_{H^+} = \sqrt{a_{H^+}\, a_{Cl^-}} = a_{HCl} \qquad (4)$$

This assumption is inexact, and further, although L_1 is completely unknown, it certainly is not 0.

We may calculate the possible magnitude of error in pa_H resulting from these two assumptions from the data already at hand. Lewis and Randall (1923, p. 382) have calculated the individual ion activity coefficients (γ_i) of some common ions from the mean ion activity coefficients (obtained from freezing point data),

employing the assumption of MacInnes that in a dilute solution of KCl $\gamma_{K^+} = \gamma_{Cl^-}$. From their table we have at 0.1 M $\gamma_{H^+} = 0.84$, $\gamma_{Cl^-} = 0.79$. In 0.1 N HCl then

$$\frac{\gamma_{H^+}}{\gamma_{Cl^-}} = 1.063 \tag{5}$$

But the mean ion activity coefficient, γ_{HCl} of 0.1 N HCl, is $10^{-1.08} = 0.832$, so that

$$\gamma_{HCl} = \sqrt{\gamma_{H^+}\gamma_{Cl^-}} = 0.832 \tag{6}$$

From these two equations by elimination of γ_{Cl^-} we get

$$\gamma_{H^+\,(0.1\ N\ HCl)} = 0.858 \tag{7}$$

i.e., the pa_H of 0.1 N HCl = 1.066, a difference of 0.014 from the assumed value of 1.08.

L_1 is estimated by Fales and Fasburgh (1918) to be 0, by Harned (1926) to be 0.00158 volt, and by Scatchard (1925) to be 0.0047 volt, equal respectively to 0, 0.025, and 0.076 pa_H units. The absolute value of the pa_H of 0.1 N HCl, and therefore of the unknown solution when 0.1 N HCl is used as the reference solution, may be in error by 0.09 pa_H units as a result of the uncertainty in the values of a_{H^+} and L_1. The estimate of this error is of course an approximation since both calculations involve the uncertain assumption of MacInnes that $\gamma_{K^+} = \gamma_{Cl^-}$.

We do not escape this dilemma by choosing another reference solution since as before its a_{H^+} and L_1 would be unknown.

Liquid Junction Potential of Unknown Solution and Reference Half-Cell.—The a_{H^+} of the unknown solution in the second cell is calculated from the relation $E_2 - e_0 = 0.0001984\ T \log a_{H^+} - L_2$. As before we are completely ignorant of the value of L_2 so that we are not in a position to calculate the true value of a_{H^+}. Thermodynamically, as Harned (1926) has shown, a liquid junction potential is given by the algebraic summation

$$L = -\Sigma \frac{RT}{N\mathbf{F}}\left[\int t_i \ln \gamma_i + \int t_i \ln c_i\right]$$

where t_i, γ_i, and c_i are the transport number, activity coefficient, and concentration of the ion. In general if $t_i = t_i' = t_i'' = \ldots$ and $\gamma_i = \gamma_i' = \gamma_i'' = \ldots$ $L \simeq 0$.
In the liquid junction

Na^+	‖	
HCO_3^-	‖	K^+
Cl^-	‖	Cl^-
H^+	‖	(In saturated KCl.)
Hb	‖	
	L_2	

since c_{H^+} is small, if we assume (1) that the transference numbers of Na^+, K^+, Cl^-, HCO_3^- are not *greatly* dissimilar and (2) that the activity coefficients of the ions in the hemoglobin solution are not *greatly* different from their respective values in simple aqueous solution, it is probably true that this liquid junction potential is small but, what is still more important, that it is practically constant over narrow ranges of concentration of (B^+, HCO_3^-, Cl^-, H^+, Hb). In other words we make the more tenable assumption that

$$L_2 = L_2' = L_2'' = \ldots . \tag{8}$$

It is evident that whereas the true pa_H of such systems may differ by < 0.09 pa_H units from the pa_H calculated on the basis of this assumption alone (*i.e.* $L_2 = 0$), the *relative* values at constant temperature in $BHCO_3$-CO_2-H_2CO_3-BCl-Hb systems, varying in the concentrations of their constituents from Hb = 0, BCl = 0, $BHCO_3$ = 10 mM to Hb = 20 mM, BCl = 200 mM, $BHCO_3$ = 50 mM, may also be in error but by a negligible factor due to the inexactness of Equation 8.

Experimental Testing of Assumption $L_2 = L_2' = L_2'' = \ldots$ by the Bjerrum Extrapolation.—We have tested this assumption over a widely varying range of $BHCO_3$, BCl, and hemoglobin concentration by means of the Bjerrum (1911) extrapolation. It suffices to say at this point that the Bjerrum extrapolation method for the determination of liquid junction potentials does not yield true liquid junction potentials but does reflect their magnitude and

would show whether there is any significant change in the junction potential of

$$\text{Saturated KCl} \;\Big\|\; \begin{matrix} \text{NaHCO}_3 \\ \text{CO}_2 \\ \text{H}_2\text{CO}_3 \\ \text{NaCl} \\ \text{Hb} \end{matrix}$$

when the system is changed from a dilute aqueous solution to a concentrated hemoglobin-containing solution.

The technique for the determination was that used by Walpole (1914). The cells used were

1. $\text{Hg}\,|\,\text{HgCl}\,|\,\text{KCl (sat.)}\,\|\,\text{KCl (}\tfrac{1}{2}\text{ sat.)}\,\|\,\text{KCl (sat.)}\,|\,\text{HgCl}\,|\,\text{Hg}$ $\qquad E_1 = \text{B}L_1$

2. $\text{Hg}\,|\,\text{HgCl}\,|\,\text{KCl (sat.)}\,\|\,\text{KCl (}\tfrac{1}{2}\text{ sat.)}\,\|\,\text{Solution X}\,\|\,\text{KCl (sat.)}\,|\,\text{HgCl}\,|\,\text{Hg}$ $E_2 = \text{B}L_2$

The first cell being symmetrical should give zero E.M.F., but, owing to the difference of the two calomel cells, gave a constant reproducible E.M.F. of -0.6 millivolt. The interposition of Solution X changed the E.M.F. so that $E_2 - E_1$ gives the Bjerrum extrapolation.

From Table I it is seen that the Bjerrum extrapolation values ($\text{B}L_2$) are all approximately equal, and since the *difference* of any pair is usually considerably less than 1 millivolt, we may say

$$\text{B}L_2 - \text{B}L_2' = \text{B}L_2' - \text{B}L_2'' < 0.001 \text{ volt} \qquad (9)$$

If then these Bjerrum extrapolation values are proportional to the liquid junction potentials of the junction

$$\text{Saturated KCl} \;\Big\|\; \begin{matrix} \text{BHCO}_3 \\ \text{CO}_2 \\ \text{H}_2\text{CO}_3 \\ \text{BCl} \\ \text{Hb} \end{matrix}$$

variations of this potential as the concentrations of the constituents are changed over the range NaHCO_3 = 10 mM; Hb, NaCl = 0

to $NaHCO_3$ = 40 mM, BCl = 160, Hb, MtHb, HbCO = 19 mM, introduce an error of less than 0.001 volt or 0.016 pa_H units in such comparative pa_H determinations.

Temperature Effect on a_{H^+} of Reference Solution and on Liquid Junction Potentials.—The mean ion activity coefficient of 0.1 N

TABLE I.

Bjerrum Extrapolation in $NaHCO_3$-H_2CO_3-Hb Systems.

Constants. June 17, 1926.
E_1 = −0.6 millivolt.
t = 38°

Solution.	$E_3 - E_1 = BL_3$
	millivolt
$NaHCO_3$, 40 mM	+0.7
Na_2CO_3, 10 " NaCl 150 mM	−0.1
HbO_2, 6.4 " base 20 "	0.0
" 6.4 " " 20 "	−0.3
" 6.4 " " 20 " NaCl 160 mM	0.0
HbCO, 18.5 " base 60 "	−0.3
" 9.2 " " 30 "	0.0
" 4.6 " " 15 "	+0.5
$NaHCO_3$, 40 "	+0.2
RHb, 18.5 "	−0.1
" 9.2 "	−0.1
MHb, 10 " base 5 mM	+0.1
" 10.0 " NaCl 150 "	−0.7

HCl as determined by Noyes and Ellis (1917) in cells without liquid junction is $\gamma_{HCl} = 10^{-1.08} = 0.832$ between 15–40°. By assuming within this temperature range that

$$\frac{\gamma_{H^+}}{\gamma_{Cl^-}} = \text{constant} \tag{10}$$

$$L_1 = \text{constant} \tag{11}$$

$$L_2 = L_2' = L_2'' = \text{constant} \tag{12}$$

we may compare calculated pa_H values at two temperatures. The magnitude of the error in such a relative comparison will depend upon the inexactness of Equations 10, 11, and 12. The temperature effect on the hydrogen ion activity of the reference

solution and the liquid junction potentials L_1 and L_2 remains, however, completely unknown although it is probably small. Nevertheless, we must not be surprised to find that thermodynamic calculations from one temperature to another based on electrometric pa_H determinations may have an inherent error (see subsequent discussion of this point).

These difficulties await solution through an exact determination of a_{H^+} in the reference 0.1 N acid at all temperatures and an adequate method for the determination of liquid junction potentials. At present the situation is a circular one. We cannot determine individual ion activities without knowing the liquid junction potential and *per contra* we cannot determine liquid junction potential without knowledge of the individual ion activities (see Harned, 1926).

Reversibility of E.M.F. of Hydrogen Electrode in $BHCO_3$-H_2CO_3-Hb Systems.—The important assumption (No. 7) that in the measurement of pa_H in $BHCO_3$-CO_2-H_2CO_3-BCl systems with or without hemoglobin, we are dealing with a reversible E.M.F. has not to our knowledge been tested experimentally. In the thermodynamic sense a reversible process is one which takes place in a system which is always in equilibrium. Hence it shows no tendency to occur spontaneously but may be made to proceed with infinite slowness in either direction by making an infinitely small change in the variables P, V, T of the system or their thermodynamic equivalents which in a galvanic cell are E, $[c]$, and T. Unless the reaction taking place within the cell is reversible, E.M.F. measurements are quite without significance, at least in so far as pa_H calculations are concerned. The cell reaction with which we are concerned in a given solution is the half reaction

$$\tfrac{1}{2}\,H_{2(P_{H_2})} \rightleftarrows H^+_{(a)} + \ominus$$

In another solution we would have

$$\tfrac{1}{2}\,H_{2(P'_{H_2})} \rightleftarrows H^+_{(a')} + \ominus$$

The total reaction in these two half-cells would then be

$$\tfrac{1}{2}\,H_{2(P_{H_2})} + H^+_{(a')} \rightleftarrows \tfrac{1}{2}\,H_{2(P'_{H_2})} + H^+_{(a)}$$

The subscripts denote the activities (in the case of gaseous hydrogen $P_{H_2} = a_{H_2}$). To prove that this cell reaction in our $BHCO_3$-

CO_2-BCl-Hb systems is reversible we must show that the free energy change ΔF is given by the equation

$$-\Delta F = NE\mathbf{F} \tag{13}$$

Since Equation 1 enables us to equate $NE\mathbf{F}$ to a, a′, P_{H_2}, and P'_{H_2} this is equivalent to saying that we must show that the E.M.F. is proportional to the logarithm of the activities of the reacting constituents; *i.e.*, H_2 or H^+.

Variation of E.M.F. of a $BHCO_3$-H_2CO_3 System with and without Hemoglobin with the Pressure of Hydrogen.—The familiar Equation 2

$$\text{E.M.F.} = 0.0001984\ T \log \frac{a_{H^+}}{a'_{H^+}} \left(\frac{P'_{H_2}}{P_{H_2}}\right)^{\frac{1}{2}}$$

enables us to apply this proof, since this relation is derived thermodynamically by making the fundamental assumption that a reversible process is concerned. Unfortunately at present we have no independent method of determining the hydrogen ion activity in our systems and naturally we cannot use an E.M.F. measurement of a_{H^+} to test itself. We may however, use variations of the molecular hydrogen activity (which is of course proportional to P_{H_2}) to test the logarithmic proportionality required for a reversible E.M.F. In any one system the a_{H^+} is constant. Therefore, with variations of hydrogen pressure

$$\text{E.M.F.} = 0.0000992\ T \log \frac{P'_{H_2}}{P_{H_2}} \tag{14}$$

To test this relation experimentally we measured the E.M.F. of one solution in equilibrium with hydrogen at widely varying pressures. For simplicity of comparison we have corrected each observed E.M.F. to P_{H_2} = 760 mm. of Hg by Equation 14. This corrected E.M.F. divided by 0.0617 gives the pa_H. These values should agree at all pressures of H_2.

Two solutions of $NaHCO_3$, one with 10 mM of carbon monoxide hemoglobin and the other without, were equilibrated at 38° with a mixture of CO_2 of about 40 mm. and hydrogen about 680 mm. The solution phase was separated over mercury without contact

with air (see technique in the following paper) and samples were transferred to electrode vessels together with gas mixtures of the *same* CO_2 tension but *varying* hydrogen tension. Nitrogen and carbon monoxide were used as diluents. The resulting E.M.F. was determined as described in the sections on technique. The data are given in Tables II and III. The close agreement of the pa_H values in the same solution over a range of hydrogen pressure of 286 to 666 mm. is a proof of the exactness with which the theoretical relation (Equation 14) is followed. It should be further noted that in the second experiment the diluent gas was

TABLE II.

Variation of E.M.F. *of Hydrogen Electrode in a $NaHCO_3$-H_2CO_3 Solution at Varying Partial Pressure of Hydrogen.*

Constants. June 2, 1926.

$NaHCO_3$ = 19.8 mM per liter. H_2CO_3 = 1.51 mM per liter.

P_{CO_2} = 47 mm. Hg. e_0 = 0.2366 volt.

α_{CO_2} = 0.0325 $\log \frac{NaHCO_3}{H_2CO_3} = 1.12$

t = 38°

N_2 used as diluent in electrode gas phase.

P_{H_2}	E_{H_2}	E.M.F. observed.	pa_H Calculated to P_{H_2} = 760 mm.Hg.
mm.Hg	*volt*	*volt*	
660	0.0019	0.6870	7.33
663	0.0018	0.6867	7.33
544	0.0045	0.6850	7.34
426	0.0079	0.6819	7.35
286	0.0131	0.6771	7.35

CO varying from 0 to 330 mm., indicating the non-interference of this gas in E.M.F. measurements. Hastings, Sendroy, Murray, and Heidelberger (1924) have previously found that a partial pressure of CO up to 10 mm. of Hg has no influence on the pa_H of phosphate buffers.

Comparison of Free Energy Change Calculated by Different Methods for the Reaction $H_2CO_3 = H^+ + HCO_3^-$.—Since the standard free energy change of a reaction is $\Delta F^0 = -RT \ln K$, this comparison merely gives a comparison of the dissociation constants of H_2CO_3 by the electrometric and other methods. We use the extrapolated E.M.F. $pK_{1\ (H_2CO_3)}$ at infinite dilution of Warburg

(1922) and of Hastings and Sendroy (1925) and the conductivity pK_1 in dilute H_2CO_3 solution of Kendal (1916).

The data in Table IV show excellent agreement at 38° between Hastings and Sendroy's pK_1 and the extrapolated conductivity

TABLE III.

Variation of E.M.F. *of Hydrogen Electrode in $NaHCO_3$-H_2CO_3-HbCO Solution at Varying Partial Pressure of Hydrogen and in Presence of CO.*

Constants. June 11, 1926.

HbCO = 11.1 mM per liter.	$\alpha_{CO_2} = 0.0266$
Available base = 34 m.-eq. per liter.	$H_2CO_3 = 1.83$ mM per liter.
NaCl = 0	$NaHCO_3 = 20.5$ m.-eq. per liter.
$P_{CO_2} = 68.8$ mm. Hg.	$e_0 = 0.2366$ volt.
$\log \frac{NaHCO_3}{H_2CO_3} = 1.05$	$t = 38°$

CO used as diluent in electrode gas phase.

P_{H_2}	P_{CO}	E.M.F. observed.	pa_H Calculated to P_{H_2} = 760 mm.Hg.
mm.Hg	*mm.Hg*	*volt*	
316	330	0.6634	7.12
442	204	0.6682	7.11
646	0	0.6736	7.12

TABLE IV.

Free Energy of Ionization of Carbonic Acid.

Method.	t	$pK_{1(H_2CO_3)}$	$\Delta F°$	$\frac{\Delta F°}{\Delta T}$
	°C.		*calories*	
E.M.F.	38	6.330	9010	16.5
	18	6.514	8680	
Conductivity.	25	6.460	8820	20.0
	0	6.656	8320	
	38*	6.360	9080	

* Extrapolated.

value, but this agreement is fortuitous since the agreement at other temperatures is lacking. Indeed $\frac{\Delta F°_t - \Delta F°_{t'}}{\Delta T}$ is quite different in the two cases. We are inclined to believe that this discrepancy is not due to any irreversibility of the E.M.F. but to the

limitation of the other assumptions under discussion and which are involved in the calculations.

Thermochemical and Electrochemical Heats of the Reaction $H_2CO_3 = H^+ + HCO_3^-$.—We have already discussed in part the difficulties involved in making thermodynamic calculations from one temperature to another from electrometric pa_H measurements. It is not surprising then to find that the heats of ionization of

TABLE V.

Heats of Ionization of Carbonic Acid and $\left(\frac{\Delta pK_1'_{(H_2CO_3)}}{\Delta T}\right)_{25°}$ *by Independent Methods.*

Method.	Heat of ionization.	$\left(\frac{\Delta pK_1'}{\Delta T}\right)_{25°}$
	calories	
Calorimetric measurement of heat of neutralization (Thomsen, 1882)	2800	0.0069
Conductivity of very dilute solution of carbonic acid at 0° and 25° (Kendal, 1916)	2940	0.0072
Electrometric $pK_1'_{(H_2CO_3)}$ in $NaHCO_3$-H_2CO_3-NaCl systems at 18° and 38° (Warburg, 1922)	2250	0.0055
(Cullen, Keeler, and Robinson, 1925)	2050	0.0050
(Stadie and Hawes, 1928)	2050	0.0050

carbonic acid determined by different methods do not agree. The van't Hoff isochore

$$\log \frac{K_t'}{K_{t'}'} = \frac{(T_1 - T_2)\ \Delta H}{2 \times 2.3\ T_1 T_2} \qquad (15)$$

enables us to calculate the heat of ionization ΔH of carbonic acid from electrochemical evaluations of K_t' and $K_{t'}'$, the dissociation constants at two temperatures. This value should agree with other determinations; *viz.*, the values of Thomsen (1882) obtained by direct calorimetric measurement of the heat of neutralization and of Kendal (1916) obtained by conductivity measurements in dilute H_2CO_3 solutions.

In Table V we have calculated $\frac{\Delta pK_1'}{\Delta T}$ at 25° by the van't Hoff isochore from the experimentally determined heats of ionization.

The electrometric method gives values 25 per cent lower than the other two methods. This is probably quite outside of the error of the methods and is due to the inexactness of our assumptions (No. II, B, 8 and 9), and also to the assumption that conductivity measures activity even in dilute solution. For the present we cannot regard this discrepancy as a proof of the irreversibility of the cell reaction. As a further test of the non-interference of hemoglobin in the reversible cell process, however, we have

TABLE VI.

$\frac{\Delta pK'}{\Delta T}$ *Calculated from* $pK_1'_{(H_2CO_3)}$ *in Solutions of Reduced Hemoglobin at 20° and 38°.*

Constants. Oct. 6, 1926.
Hb = 10.62 mM per liter.
Available base = 29.5 m.-eq. per liter.
e_0 = 0.2478 volt at 20° + (20 − t) 0.000762 volt.
0.2367 " " 38°.

t	NaCl	Γ	CO_2 tension.	Total CO_2.	H_2CO_3	E.M.F. corrected.	pa_H	pK_1'	Mean.	$\frac{\Delta pK_1'}{\Delta T}$
°C.	mM per l.	mM per l.	mm. Hg	mM per l.	mM per l.	volt				
20.1	0.0	29.5	47.2	24.08	1.99	0.6614	7.12	6.07	6.09	
21.0	0.0	29.5	48.8	24.33	2.06	0.6618	7.14	6.11		0.00467
38.0	0.0	29.5	49.4	17.74	1.32	0.6754	7.11	6.01	6.01	
38.0	0.0	29.5	65.5	19.48	1.74	0.6695	7.01	6.02		
22.8	140.0	169.5	55.3	26.35	2.07	0.6630	7.11	6.04		0.00461
38.0	140.0	169.5	52.0	18.48	1.34	0.6734	7.08	5.97		
								Mean =		0.00464

sought to show that the electrochemical heats of ionization of carbonic acid in simple aqueous solution and in hemoglobin-containing solutions are identical.

A solution of $NaHCO_3$ + NaCl of ionic strength 0.170 M was equilibrated at 38° and 20° with known CO_2 tension. The pa_H was determined electrometrically at the respective temperatures.

A solution containing hemoglobin of 10 mM concentration of approximately the same $NaHCO_3$ concentration and total ionic strength was similarly treated. The data are given in Tables

VI and VII. Technique and calculations were those outlined in the following paper. $\frac{\Delta pK_1'}{T}$ is practically the same for the two solutions; therefore the calculated heats of ionization in the two solutions are in agreement and we conclude that the identity of the calculated thermal effects of the reaction $H_2CO_3 = H^+ + HCO_3^-$ in hemoglobin- and non-hemoglobin-containing solutions is further evidence for the reversibility of the reaction in hemoglobin solutions.

Reproducibility and Constancy of the Hydrogen Electrode in 0.1 N HCl and in 10 mM Hemoglobin Solution.—The fact that platinum black in hydrogen gives a highly reproducible electrode is commonly accepted. The degree of reproducibility varies greatly

TABLE VII.

$\frac{\Delta pK_1'}{\Delta T}$ *Calculated from* $pK_1'_{(H_2CO_3)}$ *in Simple Aqueous Solution at 20° and 30°.*

Constants. Oct. 26, 1926.
$e_0 = 0.2477$ volt at 20°.
0.2367 " " 38°.
NaCl = 150 mM per liter.

t	$NaHCO_3$	CO_2 tension.	H_2CO_3	E.M.F. corrected.	p_{a_H}	pK_1'	$\frac{\Delta pK_1'}{\Delta T}$
°C.	*mM per l.*	*mm. Hg*	*mM per l.*	*volt*			
20	19.68	66.1	3.28	0.6514	6.96	6.18	0.00500
38	19.15	59.6	1.88	0.6744	7.10	6.09	

with the method of preparation of the electrode, but using our routine technique we have sought to determine the exact limits of reproducibility of the hydrogen electrode in 0.1 N HCl and in concentrated hemoglobin solutions. The data are given in Tables VIII and IX.

We obtained a series of hydrogen electrodes by replating a single platinum electrode with platinum black, using a weakly acid 5 per cent platinic chloride solution and about 6 volts for 15 to 30 seconds. Before plating, the old plating was wiped off, and the electrode soaked in cleaning fluid and thoroughly washed. The electrodes were not scraped or ignited. The electrode vessels were first flushed out with pure hydrogen and filled with the acid

TABLE VIII.

Reproducibility of the Hydrogen Electrode in 0.1 N HCl at 38°.

Series A.

Replating No.	Calomel Cell B, observed E.M.F.	Replating No.	Calomel Cell B, observed E.M.F.
	millivolts		*millivolts*
1	302.0	12	301.9
2	302.0	13	302.2
3	302.9	14	302.2
4	302.7	15	302.2
5	302.9	16	302.6
6	302.3	17	302.0
7	302.0	18	301.8
8	303.0	19	300.7
9	301.9		
10	303.1	Mean.....	302.4
11	303.1	Average deviation..	±0.4

Series B.

Replating No.	Calomel Cell M.			Replating No.	Calomel Cell M.		
	Observed.	Mean.	Deviation from probable mean.		Observed.	Mean.	Deviation from probable mean.
	millivolts	*millivolts*			*millivolts*	*millivolts*	
20	301.0			25	302.9		
	301.2	301.1	0.1		302.1		
21	301.5				300.9		
	299.7	300.6	0.4		300.1	301.5	0.5
22	301.3			26	302.1		
	301.8				301.1		
	300.6				301.0		
	300.2				300.9		
	299.8				301.6	301.3	0.3
	300.3			27	302.3		
	300.3	300.6	0.4		300.3		
23	301.2				302.0		
	301.2				301.3		
	300.8				302.4	301.7	0.3
	301.2						
	300.0			Mean		301.0	0.3
	301.0			Average deviation.		±0.5	
	300.8	300.7	0.3				
24	300.4						
	300.3						
	300.2						
	300.1	300.3	0.7				

TABLE VIII—*Concluded.*

Replating No.	Calomel Cell B.			Replating No.	Calomel Cell B.		
	Observed.	Mean.	Deviation from probable mean.		Observed.	Mean.	Deviation from probable mean.
	millivolts	*millivolts*			*millivolts*	*millivolts*	
28	302.5			31	303.9		
	302.5	302.5	0.1		303.1		
	302.9				301.9		
	303.5				301.4	302.6	0.2
	302.3			32	303.1		
	302.3				302.0		
	301.4				302.1		
	302.0				301.6		
	302.1	302.7	0.3		302.4	302.0	0.4
29	302.6			33	303.0		
	302.5				301.5		
	302.5				302.6		
	303.1				302.2		
	301.4				303.6	302.6	0.2
	302.8						
	302.5	302.5	0.1				
30	302.5						
	301.6			Mean		302.4	0.3
	301.3			Average deviation.		±0.2	
	301.3	301.9	0.5				

Summary.

Calomel half-cell.	No. of platings.	No. of refills.	Mean E.M.F.
			millivolts
B	26	53	302.4±0.3
M	8	36	301.0±0.5

or hemoglobin solution in the usual way. The readings were made at 38° after rocking for 15 to 25 minutes. In 0.1 N HCl there are 89 E.M.F. readings on thirty-four replatings. The comparisons are made with the mean of *all* the determinations (*i.e.* the probable mean value) given in the summary. In Series A only a single determination on each replating was made (excepting No. 19 which represents the mean of four refills). The average deviation from the probable mean is 0.4 millivolt. In Series B multiple determinations were performed on each replating; *i.e.*

TABLE IX.

Reproducibility of Hydrogen Electrode in 10 mM Buffered Reduced Hemoglobin at 38°.

Replating No.	Refill No.	Time from contact of electrode with Hb.		E.M.F.	Mean.	Replating No.	Refill No.	Time from contact of electrode with Hb.		E.M.F.	Mean.
		hrs.	*min.*	*millivolts*	*millivolts*			*hrs.*	*min.*	*millivolts*	*millivolts*
1	1		14	662.7		3	1		15	661.1	
			22	662.0					20	661.6	
			24	662.6					27	661.5	
			30	662.0					32	661.3	
					662.3						661.4
	2		50	661.9			2	1	5	662.1	
			57	662.1					11	662.0	
		1	3	661.9					17	662.1	
		1	10	661.5							662.1
					661.8		3	24	12	661.2	
	3	1	34	661.8					18	661.0	
		1	39	661.6					22	660.9	
		1	45	661.4							661.0
		1	50	661.4			4	24	35	661.2	
					661.6				42	661.4	
	4	3	54	661.2					50	661.2	
			59	661.2							661.3
		4	6	661.1							661.5 ± 0.1
					661.2	4	1		15	660.5	
					661.7 ± 0.3				21	661.4	
2	1		16	660.4					27	661.3	
			23	660.5					30	660.9	
			30	660.1							661.0
					660.3		2	1	10	661.5	
	2		50	661.1					15	661.5	
			56	661.7					20	661.5	
		1	4	661.8							661.5
		1	11	661.6			3	24	0	662.3	
					661.6			24	10	662.0	
	3	1	38	661.7				24	20	661.7	
		1	42	661.1							662.0
		1	49	661.8			4	24	40	662.3	
		1	54	661.8					50	662.3	
					661.8				60	662.2	
	4	3	54	662.0							662.3
		3	59	661.9							661.7 ± 0.5
		4	10	661.4							
					661.8						
					661.4 ± 0.2						

TABLE IX—*Concluded.*
Summary.

Replating No.	No. of refills.	No. of determinations.	Mean E.M.F.
			millivolts
1	4	15	661.7
2	4	14	661.4
3	4	13	661.5
4	4	13	661.1
		Mean.....	661.6 ± 0.2

the electrode vessel was washed out with water, then 0.1. N acid, and then filled afresh with acid and hydrogen. The average deviation of all the determinations from the mean is 0.5 and 0.2 millivolt respectively for the two calomel cells, while the average deviation of the mean of the determinations on one plating from the mean of all the measurements is 0.3 millivolt for both calomel electrodes. Series B, performed routinely for the determination of e_0, shows greater variation in the individual determinations than Series A in which particular care was taken. 36 per cent of single determinations in Series B, but only 16 per cent in Series A, differ by more than 0.6 millivolt from the probable mean. The points clearly established by these experiments are: (1) Our degree of reproducibility of the hydrogen electrode is about 0.6 millivolt (*i.e.*, about 0.01 pa_H). Only five out of thirty-four replatings gave E.M.F. (*i.e.* mean of one to seven refills) differing by more than 0.6 millivolt from the probable value as established by the mean of all the determinations. (2) Unless meticulous care (*e.g.* Series A) is taken one-third of *single* determinations may be more than 0.6 millivolt from the probable mean. In our routine procedure we have found that at least four determinations must be made to obtain a value within 0.5 millivolt of the mean value of a large series. For both reasons it is futile as well as unnecessary to determine e_0 for *each* electrode plating; a single determination might be in considerable error and multiple ones would be too laborious. In practice the time saved by accepting the reproducibility of the hydrogen electrode is quite appreciable. The saturated calomel cell possesses great constancy. Biweekly determinations of e_0 over a period of 2 years show an average variation from the mean of less than ± 0.3 millivolt in our cells maintained at 38°.

Our experiments with reduced hemoglobin solution were designed to test the degree of reproducibility of the hydrogen electrode in a concentrated hemoglobin solution and to determine whether replating between determinations was necessary. A stock 10 mM hemoglobin solution was buffered by the addition of phosphates to M/15 and kept at 0°. Such a heavily buffered solution would exclude changes of the pa_H of the hemoglobin solution itself so that changes in the E.M.F. would be due to variations in the electrode. Samples were equilibrated with H_2 at 38° and the E.M.F. read in the usual way. There are four replatings each with four complete refills with Hb solution and hydrogen. Comparisons are made with the mean (661.6 millivolts) of all the readings. The data given in Table IX clearly show (1) that the hydrogen electrode in hemoglobin solutions is reproducible to within 0.2 millivolt, and (2) that replating after each determination is unnecessary as indicated by average deviation of only 0.4 millivolt of the means of the determinations on each plating from the mean of all the determinations. Further, (3) the constancy of the electrode is shown by the constancy of E.M.F. even after 24 hour contact (while rocking) with the hemoglobin solution. In practice if careful inspection of the electrode reveals no protein precipitation, we have used it for two or three determinations.

Absence of Side Reactions in $BHCO_3$-H_2CO_3-Hb Systems.—In E.M.F. measurements the assumption that the cell reaction we think we are measuring and no other is the source of the E.M.F. is of major importance. In other words, we assume that no side reactions occur of sufficient magnitude to vitiate the result. The half reaction we are interested in is

$$\tfrac{1}{2}\,H_{2(P_{H_2})} \rightleftarrows H^+ \begin{pmatrix} BHCO_3 \\ H_2CO_3 \\ Hb \end{pmatrix} + \Theta \qquad (16)$$

The absence of side reactions can only be shown by the accumulated experience of many observers. For $BHCO_3$-H_2CO_3 systems the evidence at hand may be summarized as follows: (1) Identical results by different observers using different reference electrodes. Warburg (1922), Hastings and Sendroy (1925), and Stadie and Hawes (Paper IV), are in excellent agreement on the pK_1' of carbonic acid in aqueous solution. Under this head we may also

mention the work of Lepper and Martin (1926) who have shown the identity of electrometric and colorimetric pa_H of $NaHCO_3$-H_2CO_3 systems in which phosphate standards of the same ionic strength as the $NaHCO_3$ solution were used to measure colorimetric pa_H. This alone is strong indication that the objection of Evans (1926) to the use of the hydrogen electrode in bicarbonate systems is without foundation. (2) Absence of marked fluctuations or drifting. These effects are the result of irreversible or spontaneous reactions giving a variable E.M.F. We need only discuss systems containing hemoglobin, since the previous paragraph is sufficient indication of the absence of these effects in aqueous solution. As an indication of the rarity of significant E.M.F. fluctuations in hemoglobin-containing solutions, we need only mention our criteria for a valid E.M.F. measurement; *viz.*, an E.M.F. maintained constant (*i.e.* $\pm$ 1 millivolt) for 30 to 45 minutes and identical ($\pm$ 1 millivolt) values in duplicates. Practically 80 per cent of the determinations reported in the accompanying papers would meet these (for Hb solutions) rigid requirements. The remaining 20 per cent, mostly early determinations, falls somewhat outside of this but is included since we believe it to be without significant error. Moreover, using hemoglobins made by different methods and over a time period of more than a year, we have repeatedly obtained identical results (for the same thermodynamic environment) for the value of pK_1' of carbonic acid.

Hydration of Hydrogen Ion in Hemoglobin Solutions.—There is sufficient evidence to show that a considerable fraction of the hydrogen ions in aqueous solutions is hydrated and exists as an ion having the composition H_3O^+. The relation of this fact to pa_H determinations in concentrated hemoglobin solution is made clear by the following considerations. The equilibrium between the hydrogen electrode and the solution is one between unhydrated ions since hydrated ions do not exist in the electrode; *i.e.*, the hydrogen electrode measures the activity of unhydrated ions. From Equation 1 when $a'_{H^+} = 1$ and $P_{H_2} = 1$ atmosphere we get

$$\frac{E - E_0}{\frac{RT}{NF}} = pa_H \qquad (17)$$

where E_0 is the E.M.F. of the normal hydrogen electrode. The equilibrium between hydrated ions, unhydrated ions, and water is given by

$$\frac{a_{H_3O^+}}{a_{H^+} \times a_{H_2O}} = \frac{a'_{H_3O}}{a'_H \times a'_{H_2O}} = k = 1 \quad (18)$$

We adopt the convention for hydrates and let $k = 1$. As before, since in the normal hydrogen electrode

$$a'_{H^+} = 1,\ a'_{H_3O^+} = 1, \text{ and } a'_{H_2O} = 1$$

$$pa_{H_3O^+} = pa_H - \log a_{H_2O} \quad (19)$$

Whether to use Equation 17 or 19, *i.e.* whether to calculate pa_H or $pa_{H_3O^+}$, depends upon the nature of the chemical process under investigation. In most equilibria studies, the reaction is one involving H^+ and not H_3O^+. In the present problem, for example, the reaction under consideration is $H^+ + HCO_3^-$. Therefore pa_H should be calculated. In many kinetic problems, however, H_3O^+ is the reacting ion and proper comparisons from solution to solution require the calculation of $pa_{H_3O^+}$ by Equation 19. In the next paper, however, we will show how for a quite different reason the activity of water, which in our most concentrated Hb-salt solutions is diminished to 0.98, enters into the calculation of the activity coefficient of the bicarbonate ion.

SUMMARY AND CONCLUSIONS.

1. The assumptions necessary to calculate the pa_H in $BHCO_3$-CO_2-H_2CO_3-BCl-Hb systems from E.M.F. measurements with the hydrogen electrode are discussed.

2. The absolute pa_H measured electrometrically is in error by an indeterminate amount due to faulty assumptions concerning the a_{H^+} of the reference solution and the liquid junction potentials 0.1 N HCl, saturated KCl, and

$BHCO_3$	‖	
BCl	‖	Saturated KCl
Hb	‖	

Approximate evaluations of this error are made.

3. The liquid junction potential between solutions of $BHCO_3$-CO_2-H_2CO_3-BCl-Hb of widely varying concentrations and saturated KCl is probably negligible as indicated by the Bjerrum extrapolation. The error in relative pa_H determinations in such solutions is probably < 0.016 pa_H units.

4. The hydrogen electrode is a reversible source of E.M.F. in $BHCO_3$-CO_2-H_2CO_3-BCl-Hb systems as shown by its adherence to the relation E.M.F. $\propto \frac{RT}{NF} \ln P_{H_2}$ over a wide range of hydrogen pressure. CO in high concentration does not alter this reversibility.

5. The free energies of the ionization of H_2CO_3 calculated from E.M.F. and conductivity measurements are in good agreement at 38° but not identical at all temperatures.

6. The heats of ionization of H_2CO_3 calculated from thermal and conductivity data (which agree) and from E.M.F. data differ by 20 per cent from each other, but when measured by E.M.F. alone are identical in simple aqueous solution and 10 mM hemoglobin solution.

7. Appreciable side reactions giving rise to irreversible E.M.F. are absent in $BHCO_3$-CO_2-H_2CO_3-BCl-Hb systems.

8. The hydrogen electrode is reproducible ($\pm$ 0.5 millivolt) in 0.1 N HCl and in 10 mM hemoglobin solution.

9. There is sufficient evidence at hand to establish the reversibility of the cell reaction in E.M.F. measurements of a_{H^+} in $BHCO_3$-CO_2-H_2CO_3-BCl-Hb systems.

10. The relation of hydrated and unhydrated hydrogen ions in hemoglobin solutions is discussed.

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STUDIES ON THE OXYGEN-, ACID-, AND BASE-COMBINING PROPERTIES OF BLOOD.

IV. THE APPARENT FIRST DISSOCIATION CONSTANT, pK_1', OF CARBONIC ACID AND THE ACTIVITY COEFFICIENT OF THE BICARBONATE ION IN SOLUTIONS OF HEMOGLOBIN, METHEMOGLOBIN, CYANHEMOGLOBIN, AND NITRIC OXIDE HEMOGLOBIN AT VARYING IONIC STRENGTHS.

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INTRODUCTION.

The importance of the apparent first dissociation constant pK_1' of carbonic acid, particularly in the acid-base equilibrium of the blood, has prompted extensive work on its evaluation both in pure aqueous and protein-containing solutions. The proteins most frequently used were those of blood serum or plasma, and, except for some isolated observations by Hasselbalch (1916) on dialyzed hemoglobin and by Warburg (1922) and Van Slyke, Hastings, Murray, and Sendroy (1925) on hemolyzed red blood cells and on one solution of hemoglobin, no systematic study of pK_1' in hemoglobin-containing solutions has been reported. In this paper we report the results of a study of the influence on $pK_1'_{(H_2CO_3)}$ and the activity coefficient, $\gamma_{HCO_3^-}$, of the bicarbonate ion of varying concentrations of hemoglobin and its derivations, *viz.* reduced hemoglobin, carbon monoxide hemoglobin, methemoglobin, nitric oxide hemoglobin, and cyanhemoglobin, in solutions whose salt content was varied by the addition of NaCl.

Activity Coefficient of the Bicarbonate Ion and the Debye-Hückel Theory.—The concept of activity was first applied to $BHCO_3$-H_2CO_3 systems by Warburg (1922) and subsequently by Hastings and Sendroy (1925). Warburg emphasized the importance of calculating the hydrogen ion concentration in activity units subsequently denoted (exponentially) pa_H by Sørensen and Linderstrøm-Lang (1924), and he further showed that the activity

coefficient, $\gamma_{HCO_3^-}$, of the bicarbonate ion and therefore pK_1' varied with changing salt concentration. Warburg found the relation $\log \gamma_{HCO_3^-} = -k\sqrt[3]{c}$ developed by Bjerrum and Gjaldbaek (1919) where c is total (monovalent) salt concentration and k is a constant ($= 0.46$) to hold quite exactly for aqueous solution up to $c = 0.4$ M.

The study of the variation of the activity coefficient of an ion in the presence of a mixture of electrolytes in water and other solvents received considerable impetus by the work of Debye and Hückel (1923) who derived theoretically an expression relating the activity coefficient of any given ion to its valence and the properties of the solvent. The Debye-Hückel theory will be discussed in detail in the following paper. We need here give only the limiting equation for uni-univalent salts where

$$\log \gamma_{HCO_3^-} = -0.53\sqrt{\Gamma} \quad .$$

Γ = ionic strength in mols per liter = $\frac{1}{2}\Sigma z_i^2 [c_i]$.

(z_i = valence of the ion.)

which in general holds only up to about $\Gamma = 0.010$ M. Hastings and Sendroy (1925), however, calculated $\log \gamma_{HCO_3^-}$ from determinations of $pK_1'_{(H_2CO_3)}$ at varying $NaHCO_3$ and NaCl concentrations and found that the limiting law was followed not only in dilute solutions, but contrary to the general experience with the *mean ion activity coefficient* of other salts the law held to concentrations as high as 0.18 M. They recalculated Warburg's results which were in excellent agreement with their own. The combined data gave $\log \gamma_{HCO_3^-} = -0.53\sqrt{\Gamma}$. It will be noted that 0.53 is the theoretical value for water at 38°.

Apparent First Dissociation Constant of Carbonic Acid and the Activity Coefficients of the Bicarbonate Ion.—Let

a, a_i = activity of a molecule or ion.

(HCO_3^-) = stoichiometrical concentration of HCO_3^- ion in mM per kilo of H_2O.

$\gamma_{HCO_3^-} = \dfrac{a_{HCO_3^-}}{(HCO_3^-)}$, activity coefficient of HCO_3^- ion.

Γ = ionic strength = $\frac{1}{2}\Sigma z_i^2 [c_i]$; *i.e.*, half the sum of the concentration of each ion in the solution times its valence squared. z_i is the valence and $[c_i]$ the concentration of the ion in *mols per liter*. (In the tables Γ is given in mM per liter.)

In an aqueous solution in equilibrium with a gas phase containing CO_2 at a partial pressure P_{CO_2}, we have the molecular or ionic species H^+, HCO_3^-, and a series of hydrates. Of the possible hydrates, CO_2, H_2O, $CO_2 \cdot 2H_2O$ . . . . $CO_2 \cdot nH_2O$ in solution, we will consider the first only, *i.e.* H_2CO_3. The reaction of it with H^+ and HCO_3^- is $H_2CO_3 \rightleftarrows H^+ + HCO_3^-$ from which the equilibrium constant is

$$\frac{a_{H^+}\, \gamma_{HCO_3^-}\, (HCO_3^-)}{a_{H_2CO_3}} = K_1^0 \qquad (1)$$

Let

$$h = \frac{(H_2CO_3)}{\Sigma^n\, (CO_2 \cdot nH_2O)}$$

be the *stoichiometrical* fraction of total physically dissolved CO_2 in all forms existing as H_2CO_3. No restriction is placed on the value of h. Then if $(\alpha^s_{CO_2})$ is the stoichiometrical solubility coefficient of CO_2 in the given *solution* in M per kilo of H_2O

$$a_{H_2CO_3} = h\, \gamma_{H_2CO_3}\, (\alpha^s_{CO_2})\, P_{CO_2} \qquad (2)$$

To evaluate $a_{H_2CO_3}$ we have the equilibrium, CO_2 (gas) + H_2O = H_2CO_3, whose equilibrium constant is given by

$$\frac{a_{H_2CO_3}}{a_{H_2O}\, P_{CO_2}} = k$$

From which we get in the case of the given solution

$$\frac{h\, \gamma_{H_2CO_3}\, (\alpha^s_{CO_2})}{a_{H_2O}} = k$$

whereas in water, since $h = h_0$, $\gamma_{H_2CO_3} = 1$, $(\alpha^s) = \alpha^0$, and $a_{H_2O} = 1$, we have $h_0\, \alpha^0_{CO_2} = k$. These two equations give

$$h\, \gamma_{H_2CO_3} = h_0\, \frac{\alpha^0_{CO_2}}{(\alpha^s_{CO_2})}\, a_{H_2O}$$

In dilute solutions with little error a_{H_2O} is constant and = 1 (see below). Then from Equation 2, $a_{H_2CO_3} = h_0 \alpha^0_{CO_2} P_{CO_2}$, so that Equation 1 becomes

$$\frac{a_{H^+} \gamma_{HCO_3^-} (HCO_3^-)}{h_0 \alpha^0_{CO_2} P_{CO_2}} = K_1^0$$

which is the *true* first dissociation constant of H_2CO_3. In this equation h_0, α^0, and K_1^0 are constants but h, (α^s), and $\gamma_{H_2CO_3}$ are variables.

It is clear then that while thermodynamic methods can evaluate $\frac{h}{h_0}\gamma_{H_2CO_3}$ (merely by means of solubility measurements) they can never measure h, h_0, or $\gamma_{H_2CO_3}$ separately or in any pair. To determine h and h_0 use must be made of other methods; *e.g.*, the kinetic methods of Faurholt (1924–25). h must be known at varying salt concentration in order empirically to extrapolate to pure water to obtain h_0. These data are not at hand, but Faurholt (1924–25) found h in a moderately concentrated salt solution to be about 0.0015. Using the hydrate convention, since h_0 is a constant (at constant T), we can let $h_0 = 1$ without extrathermodynamic assumption. Then

$$\frac{a_{H^+} \gamma_{HCO_3^-} (HCO_3^-)}{\alpha^s_{CO_2} P_{CO_2}} = K_1$$

where K_1 is the *apparent* first dissociation constant of H_2CO_3. It is customary to equate the stoichiometrical concentrations of the preceding equation to quotients of constants and activity coefficients. Thus the equation gives

$$\frac{a_{H^+} \gamma_{(HCO_3^-)}}{\alpha^s_{CO_2} P_{CO_2}} = \frac{K_1}{\gamma_{HCO_3^-}} = K_1' \qquad (3)$$

It is important to emphasize that K_1 is a thermodynamic equilibrium constant whereas K_1' is a variable.

Calculation of the Bicarbonate Ion Activity Coefficients γ_0 and γ_*.—$\gamma_{HCO_3^-}$ measures the total effect of the environment upon the bicarbonate ion and indicates to what extent the ion has departed

from ideal behavior in the solution. It is easy to show thermodynamically that $\gamma_{HCO_3^-}$ may be split into as many activity coefficients as there are factors causing this departure from the ideal state. In our hemoglobin solutions, we will consider two factors only to be of significance: (1) the interionic effect of salt denoted by γ_0; (2) the interaction of hemoglobin denoted by γ_*.

By definition

$\gamma_0 = 1$ when concentration of electrolytes = 0.
$\gamma_* = 1$ " " " hemoglobin = 0.

Also

$$\gamma_{HCO_3^-} = \gamma_0 \gamma_*$$

The extrapolated value of pK_1' at constant finite hemoglobin concentrations and $\Gamma = 0$ we call pK_*. By Equation 2 $\frac{K_1}{\gamma_* \gamma_0} = K_1'$, and since $\gamma_0 = 1$ and $K_1' = K_*$ when $\Gamma = 0$, we get $\frac{K_1}{\gamma_*} = K_*$, or

$$\log \gamma_* = pK_* - pK_1 \tag{4}$$

γ_* being known, γ_0 may be calculated at finite values of Γ and the same concentration of hemoglobin by the equation

$$\log \gamma_0 = \log \gamma_{HCO_3^-} - \log \gamma_* \tag{5}$$

We have split $\gamma_{HCO_3^-}$ into two activity coefficients which may thus be readily calculated from our data. In the subsequent paper we will show that γ_* and γ_0 may be calculated by the Debye-Hückel theory and that our data are in harmony with, though not necessarily a proof of the validity of the theory in our solutions.

Assumptions Necessary to Evaluate $\gamma_{HCO_3^-}$.—Up to this point no extra thermodynamic assumptions have been made. To determine a_{H^+}, (HCO_3^-), $\gamma_{HCO_3^-}$, and K_1, the apparent first dissociation constant of carbonic acid, several assumptions must be made which should be clearly appreciated.

a_{H^+} *Determinations.*—The assumptions made in measuring a_{H^+} have been discussed in the preceding paper and we will assume that the a_{H^+} determinations in our systems are valid.

Determination of (HCO_3^-).—To measure (HCO_3^-) we must make two assumptions of distinctly different characters. The conceivable combinations of CO_2 in our hemoglobin solutions are (stoichiometrically in mols per kilo of H_2O):

$$(\text{Total } CO_2) = (BHCO_3) + (CO_2)\text{aq.} + (CO_2, nH_2O) + (X_{HbCO_2}) \quad (6)$$

$(BHCO_3)$ = any bicarbonate. B = Na or Hb.
(CO_2)aq. = CO_2 in solution.
(CO_2, nH_2O) = hydrates of CO_2, $n = 1, 2, \ldots$.
(X_{HbCO_2}) = any undetermined CO_2 combination with Hb not bicarbonate.

Although there is no conclusive evidence we will assume $(X_{HbCO_2}) = 0$. The solubility coefficient $(\alpha^o_{CO_2})$ of CO_2 in our solutions gives

$$(\alpha^o_{CO_2})\, P_{CO_2} = (CO_2)\text{ aq.} + \Sigma\,(CO_2, nH_2O)$$

We then may calculate $(BHCO_3)$ by Equation 6 and in conformity with the present concept of strong electrolytes we assume the bicarbonate in whatever form to be completely ionized. Two forms may exist, *viz.* $NaHCO_3$ and $HbHCO_3$. The assumption applies to both forms and subsequently we will present some experimental evidence bearing on it. Hence $(HCO_3^-) = (BHCO_3)$. Equation 3 then becomes in log form

$$pK_1' = pa_H - \log \frac{(BHCO_3)}{\alpha^o_{CO_2} P_{CO_2}} \quad (7)$$

and from Equation 3 $\dfrac{K_1}{\gamma_{HCO_3^-}} = K_1'$ or

$$pK_1' = pK_1 + \log \gamma_{HCO_3^-} \quad (8)$$

Calculation of pK_1 *at* $\Gamma = 0$ *by Extrapolation.*—In pure water we adopt the convention that $\gamma_{HCO_3^-} = 1$ when $\Gamma = 0$; hence Equation 8 enables us to calculate $\gamma_{HCO_3^-}$ at finite values of Γ if we know pK_1 at $\Gamma = 0$. It is necessary to employ graphical methods in such an extrapolation by plotting experimental pK_1' against some arbitrary function of the concentration. Usually such an extrapolation is accomplished with considerable difficulty and with the possibility of error. Fortunately in the case of $NaHCO_3$-NaCl systems in *water* this task appears to be easy. The data of

Warburg, Hastings and Sendroy, and ourselves show that the plot of pK_1' against $\sqrt{\Gamma}$ gives an exactly linear relation between the concentrations $\Gamma = 0.010$ M and 0.3 M. Although the region $\Gamma = 0$ to 0.010 M is not necessarily ideal, the fact that the slope β of the $pK_1' - \sqrt{\Gamma}$ line is the theoretical slope β_0 of the limiting law of Debye and Hückel, gives considerable certainty to the extrapolation through this region.

In our Hb, $NaHCO_3$, NaCl solutions, as we shall show, straight line relations are also found between pK_1' and $\sqrt{\Gamma}$ from $\Gamma = 0.020$ to 0.5 M or higher. On experimental grounds then and for the theoretical reasons developed in the next paper we have equal assurance of the validity of the extrapolation from 0.020 to 0.

Activity of Water in Concentrated Hemoglobin Solutions.—The activity of the water, which in the development of Equation 3 = 1, may be determined from a knowledge of the vapor pressure or the freezing point depression Δ of the solution. We may use the equation (Lewis and Randall, 1923, p. 284)

$$\log a_{H_2O} = -0.0042\,\Delta \qquad (9)$$

as a sufficient approximation. We have measured Δ on the most concentrated solutions used and found it to be about 1.5°. Thus the minimum value of $a_{H_2O} = 0.983$; *i.e.*, practically constant and = 1.

Variations of pK_1 of Carbonic Acid in Aqueous Solutions of Varying Concentrations of Reduced Hemoglobin, Carbon Monoxide Hemoglobin, Methemoglobin, Nitric Oxide Hemoglobin, and Cyanhemoglobin at Varying Ionic Strengths.—In general our procedure has been to equilibrate in glass saturators at 38° solutions of known concentrations of hemoglobin or its derivatives, known total available base, $NaHCO_3$ and NaCl, with known gas phases; to determine the pa_H electrometrically and to calculate $pK_1'_{(H_2CO_3)}$ pK_*, log $\gamma_{HCO_3^-}$, log γ_*, and log γ_0 by means of Equations 4, 5, 7, and 8. The results are summarized in graph form (Figs. 1 to 13), the complete data being given in Tables 1 to 13. The technique and the method of calculation employed are as follows:

Experimental Methods.

Preparation of Hemoglobin Derivatives.—Fresh, washed horse red blood cells were electrodialyzed for the preparation of crystalline hemoglobin

according to the method of Stadie and Ross (1926). Crystalline carbon monoxide hemoglobin, methemoglobin, cyanhemoglobin, and nitric oxide hemoglobin were prepared by a modification of this method, the details of which will appear in a subsequent paper.

The crystals were dissolved in the requisite amount of alkali to make a concentrated solution (15 to 20 mM) which was then analyzed for total available base by the Stadie and Ross (1925) method and for total pigment by the Stadie (1920) method. Where necessary the oxygen capacity was determined by the Van Slyke and Neill (1924) manometric method. The solution was diluted as required and NaCl was added by weight to a known volume to obtain solutions of given ionic strengths.

Saturation.—The saturators were of the form described by Austin *et al.* (1922). They were exhausted and filled with hydrogen four or five times on a gas manifold to displace the air, and the solution was then run in together with the approximate amount of CO_2 necessary to give the pressure desired. Hydrogen was used as the indifferent gas. The saturation of 30 to 45 minutes duration was usually repeated three times, to approximate more closely the desired conditions. When carbon monoxide hemoglobin was used, a small (10 to 20 mm.) pressure of carbon monoxide gas was used in the successive saturations. The temperature of the water bath used was 38° ± 0.1°.

Measurement of Gas Phase.—The saturator was clamped to a stand with upper stop-cock out of water. A small mercury manometer was attached, the stop-cock momentarily opened, and the pressure, which was always in slight excess of atmospheric pressure, measured. After separation of the solution phase, analysis of the gas phase by the Haldane gas analyzer gave the percentage of CO_2. The calculation (barometer + excess pressure − aqueous tension) times per cent CO_2 gives the partial pressure of CO_2.

Solution Phase.—This was separated at 38° out of contact with air by connecting the lower stop-cock of the saturator to a mercury receiver in the water bath and drawing the solution into the receiver by slight negative pressure. 1 cc. sample was then analyzed for total CO_2 by the Van Slyke and Neill (1924) manometric method.

Electrometric p_{aH} Measurement.—The electrode vessel used was of the Clark-Cullen type, equipped with thermometer and of 2.5 cc. capacity. It was washed out with 500 cc. of a CO_2-H_2 mixture made up to give the CO_2 pressure at 38° found in the final saturation. The solution was then run directly into the electrode from the mercury receiver, leaving a gas bubble about 0.1 to 0.2 cc. in volume. The electrode was quickly warmed up to 38° over a Bunsen flame and then rocked in an air bath at 38° ± 0.1°. Duplicates were made in all cases. The reference half-cell was a saturated calomel cell maintained at 38° and standardized against 0.1 N HCl to which an activity value of 1.08 was assigned. e_0 of this half-cell was calculated by the formula

$$e_0 = \text{E.M.F.} - 1.08 \times 0.0617 \qquad (10)$$

From the E.M.F. of the cell

$$\text{Hg} \left| \text{HgCl} \right| \begin{matrix}\text{KCl} \\ \text{saturated}\end{matrix} \left| \begin{matrix}\text{Saturated KCl} \\ \text{bridge}\end{matrix} \right\| \begin{matrix}\text{Unknown} \\ \text{solution}\end{matrix} \left| \text{H}_2 \right| \text{Pt}$$

the pa_H of the unknown was calculated by the equation

$$pa_H = \frac{\text{E.M.F.} - e_0}{0.0617} \tag{11}$$

All potentials were corrected to 760 mm. of dry hydrogen by the equation

$$E_{H_2} = 0.03089 \log \frac{760}{P_{H_2}} \text{ volts}$$

The potentiometer was a Leeds and Northrup type K used with a Leeds and Northrup galvanometer of the d'Arsonval type. A Marion Eppley unsaturated Weston cadmium cell standardized by the United States Bureau of Standards was used.

The connecting salt bridge was a saturated KCl solution in 2 per cent agar. This served excellently to prevent mechanical mixing of KCl solution with the unknown.

Calculations.—The molecular concentration of the hemoglobin or its derivatives was calculated from the total hemoglobin by assuming 1 molecule of oxygen equivalent to 1 molecule of hemoglobin. The concentration of H_2CO_3 in water at 38° was taken as $0.0326 \times P_{CO_2}$ mM per liter. In salt solution it was assumed to be depressed linearly 20 per cent per mol of Γ (Hastings and Sendroy, 1925). In hemoglobin solution its solubility was assumed to be proportional to the water present (Van Slyke, Hastings, Murray, and Sendroy, 1925) so that

$$[H_2CO_3] = 0.0326\, P_{CO_2}\, ([W] - 0.2\ \Gamma) \tag{12}$$

A large number of dry weight determinations on crystallized hemoglobin solutions of varying concentration gave a straight line relation between total solids and concentration, whose equation was

$$\text{Weight per cent Hb} = 1.6 \text{ mM Hb per liter}$$

which was used to calculate kilos of H_2O per liter.

$[W]$ = kilos H_2O per liter = D^* − 0.016 [Hb] − 0.000059 [NaCl].
[NaCl] = NaCl mM per liter.
$[CO_2]$ = total CO_2 mM per liter.
$[H_2CO_3]$ = dissolved CO_2 mM per liter (Equation 12).
$[NaHCO_3]$ = [total CO_2] − $[H_2CO_3]$ mM per liter.

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* D = density assumed 1 throughout. This assumption makes pK_1' too small by 0.03 and 0.015 when Hb is 20 and 10 mM per liter respectively, a sufficient approximation.

$$[NaHCO_3] = \frac{[NaHCO_3]}{[W]} = \text{mM per kilo } H_2O.$$

[NaHb] = [available base] − [$NaHCO_3$] mM per liter.

pK_1' was calculated by Equation 7. The ionic strength $\Gamma = \frac{1}{2} \Sigma z^2 [c_i]$ was calculated *per liter of solution* by assuming a valence of 1 for the hypothetical hemoglobin ion of sodium hemoglobinate, so that

$$\Gamma = [NaHb] + [NaHCO_3] + [NaCl]$$
$$= [\text{available base}] + [NaCl]$$

In the figures Γ is in mols per liter.

TABLE I (*Fig. 1*).

$pK_1'_{(H_2CO_3)}$ in Aqueous Solution of $NaHCO_3$-H_2CO_3-NaCl at Varying Ionic Strength.

Constants. Nov. 16, 1925.
$t = 38°$
$e_0 = 0.2366$ volt.

$NaHCO_3$	NaCl	Γ	P_{CO_2} tension.	H_2CO_3	E.M.F. corrected.	pa_H
mM per l.	*mM per l.*	*mM per l.*	*mm.Hg*	*mM per l.*	*volt*	
30.00	0	30.0	53.3	1.73	0.6970	7.46
30.00	27.6	57.6	43.8	1.41	0.7016	7.54
30.00	66.0	96.0	32.0	1.02	0.7062	7.61
10.52	120.0	150.0	31.4	0.99	0.7054	7.60
10.52	0	10.5	95.5	3.11	0.6552	6.79
10.52	0	10.5	94.3	3.07	0.6573	6.82
19.41	0	19.4	69.7	2.26	0.6797	7.18
19.12	150.0	169.1	59.6	1.91	0.6744	7.10

DISCUSSION AND RESULTS.

$pK_1'_{(H_2CO_3)}$ in Aqueous Solution.—Fig. 1 (Table I) shows the plot of pK_1' against $\sqrt{\Gamma}$ in aqueous solutions of 10, 20, and 30 mM of $NaHCO_3$ with NaCl added up to a concentration of $\Gamma = 170$ mM. The equation for the best line through the points is

$$pK_1' = 6.330 - 0.54 \sqrt{\Gamma} \quad (13)$$

which is in excellent agreement with the data of Warburg and Hastings and Sendroy. Therefore we may write by Equation 8 since $pK_1 = 6.330$

$$-\log \gamma_{HCO_3^-} = 0.54 \sqrt{\Gamma} \quad (14)$$

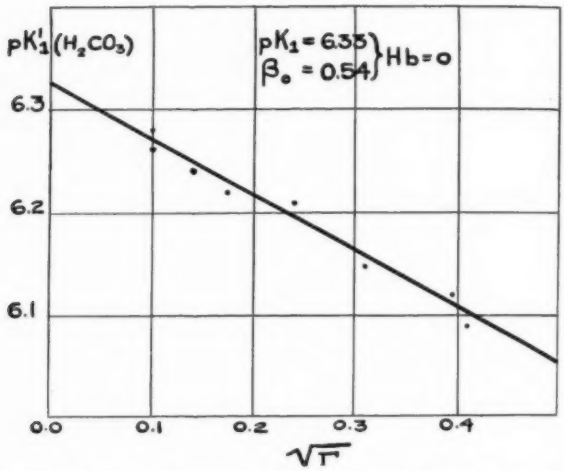

FIG. 1. $pK_1'_{(H_2CO_3)}$ in aqueous solution of $NaHCO_3$-H_2CO_3-NaCl at varying ionic strength.

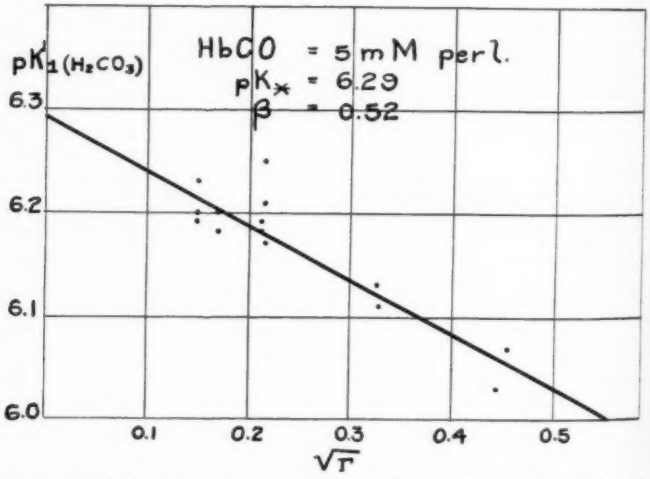

FIG. 2. $pK_1'_{(H_2CO_3)}$ in 5 mM carbon monoxide hemoglobin solution at varying ionic strength.

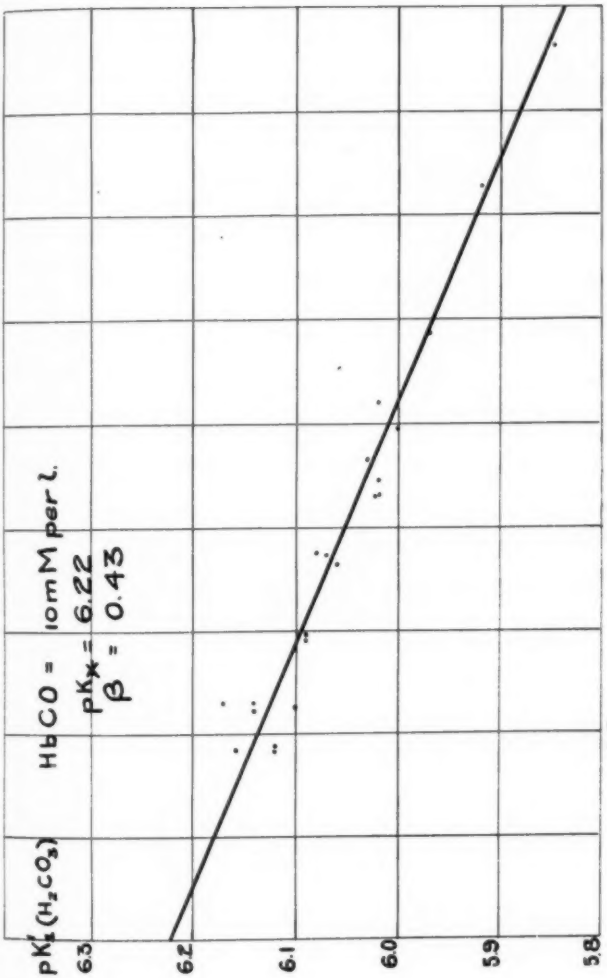

FIG. 3. pK_1' (H_2CO_3) in 10 mM carbon monoxide hemoglobin solution at varying ionic strength.

$pK_1'_{(H_2CO_3)}$ in Solutions of HbCO at Varying Ionic Strength.— There are three series of determinations on carbon monoxide hemoglobin solutions in which the HbCO concentration is ap-

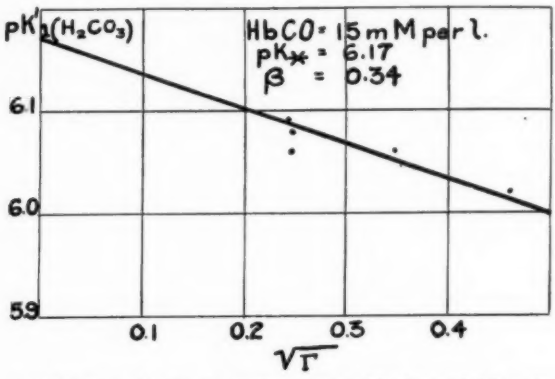

FIG. 4. $pK_1'_{(H_2CO_3)}$ in 15 mM carbon monoxide hemoglobin solution at varying ionic strength.

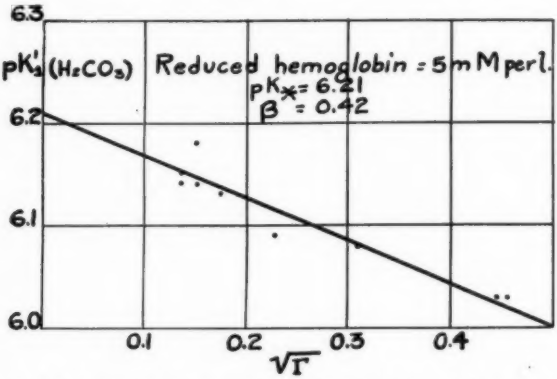

FIG. 5. $pK_1'_{(H_2CO_3)}$ in 5 mM reduced hemoglobin solution at varying ionic strength.

proximately 5, 10, and 15 mM respectively (Tables II to IV, Figs. 2 to 4). The available base varies from 22 to 60 mM, added NaCl from 0 to 700 mM, with a variation in ionic strength from 22 to

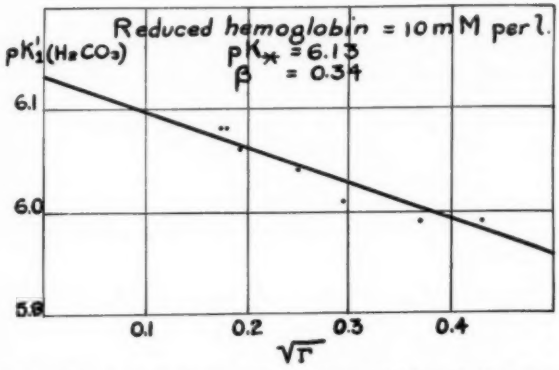

FIG. 6. $pK_1'_{(H_2CO_3)}$ in 10 mM reduced hemoglobin solution at varying ionic strength.

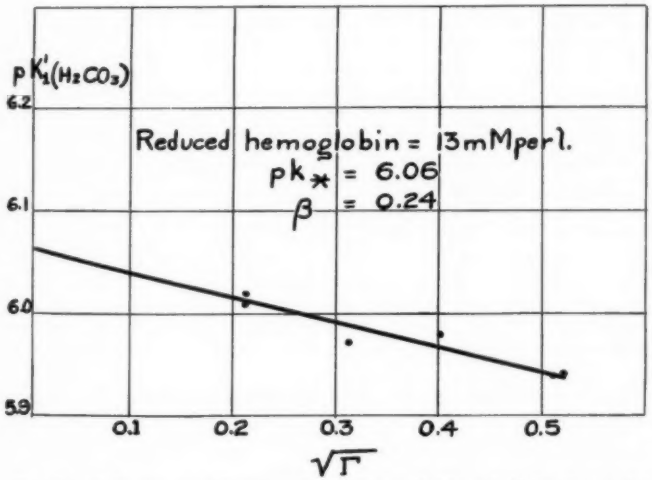

FIG. 7. $pK_1'_{(H_2CO_3)}$ in 13 mM reduced hemoglobin solution at varying ionic strength.

744 mM. When the pK_1' values are plotted against $\sqrt{\Gamma}$ (Figs. 2, 3, and 4) linear relations are apparently seen best in the twenty-one point experiment at HbCO = 10 mM but are obvious at the other

concentrations. We may conclude that the relation of pK_1' and ionic strength in HbCO-containing solutions has the same form as in simple aqueous solution; *i.e.*, the limiting law of Debye and Hückel which for our present purpose is written

$$pK_1' = pK_* - \beta \sqrt{\Gamma} \qquad (14,a)$$

TABLE II (*Fig. 2*).

$pK_1'_{(H_2CO_3)}$ in 5 mM Carbon Monoxide Hemoglobin Solution at Varying Ionic Strength.

Constants.

$t = 38°$

$e_0 = 0.2366$ volt.

Date.	Total Hb.	Available base.	NaCl	Γ	CO_2 tension.	H_2CO_3	$NaHCO_3$	E.M.F. corrected.	p_H
1926	*mM per l.*	*mM per l.*	*mM per l.*	*mM per l.*	*mm. Hg*	*mM per l.*	*mM per l.*	*volt*	
May 14	5.94	29.1	0	29.1	42.5	1.24	15.38	0.6870	7.30
" 14	5.94	29.1	0	29.1	42.2	1.23	15.49	0.6864	7.29
" 14	5.94	29.1	80	109.0	41.7	1.19	15.42	0.6821	7.22
" 14	5.94	29.1	170	199.0	39.8	1.12	15.90	0.6802	7.19
" 20	4.49	22.5	0	22.5	30.9	0.927	10.21	0.6858	7.28
" 20	4.49	22.5	0	22.5	32.7	0.981	10.94	0.6830	7.24
" 20	4.49	22.5	0	22.5	32.2	0.966	10.43	0.6829	7.24
" 20	4.49	22.5	85	108.0	31.9	0.937	10.57	0.6803	7.19
" 20	4.49	22.5	185	208.0	32.2	0.927	11.07	0.6776	7.15
June 7	5.78	47.0	0	47.0	18.1	0.528	28.03	0.7259	7.93
" 7	5.78	47.0	0	47.0	23.9	0.698	28.92	0.7165	7.78
" 8	5.78	47.0	0	47.0	52.5	1.53	31.39	0.7030	7.56
" 9	5.92	45.2	0	45.2	42.7	1.25	30.33	0.7038	7.57
" 9	5.92	45.2	0	45.2	43.2	1.26	30.47	0.7029	7.56

The values of pK_* may be obtained by extrapolation to $\Gamma = 0$, $\log \gamma_*$ calculated by Equation 4, and the β values calculated from the slopes. We have

HbCO per liter. mM		
5	$pK_1' = 6.29 - 0.52 \sqrt{\Gamma}$	(15)
	$\log \gamma_* = -0.04$	(16)
10	$pK_1' = 6.22 - 0.42 \sqrt{\Gamma}$	(17)
	$\log \gamma_* = -0.11$	(18)
15	$pK_1' = 6.17 - 0.34 \sqrt{\Gamma}$	(19)
	$\log \gamma_* = -0.16$	(20)

TABLE III (*Fig. 3*).

$pK_1'_{(H_2CO_3)}$ in 10 mM Carbon Monoxide Hemoglobin Solution at Varying Ionic Strength.

Constants.

$t = 38°$

$e_0 = 0.2366$ volt.

Date.	Total Hb.	Available base.	NaCl	Γ	CO_2 tension.	H_2CO_3	$NaHCO_3$	E.M.F. corrected.	pa_H
1925	*mM per l.*	*mM per l.*	*mM per l.*	*mM per l.*	*mm. Hg*	*mM per l.*	*mM per l.*	*volt*	
Dec. 16	10.92	34.0	0	34.0	37.5	0.99	9.51	0.6740	7.10
" 16	10.92	34.0	0	34.0	50.9	1.34	11.06	0.6692	7.03
" 16	10.92	34.0	150	184.0	43.5	1.11	11.34	0.6695	7.03
" 21	10.20	33.8	236	269.0	47.3	1.20	16.25	0.6775	7.16
" 22	10.20	33.8	150	184.0	55.0	1.42	17.39	0.6749	7.11
" 22	10.20	33.8	100	134.0	49.7	1.30	16.40	0.6777	7.16
" 29	10.16	33.4	0	33.4	49.2	1.32	17.29	0.6833	7.27
" 29	10.16	33.4	50	88.4	50.7	1.34	17.75	0.6812	7.22
" 29	10.16	33.4	105	138.4	49.2	1.29	18.21	0.6814	7.22
" 31	12.20	50.9	147	198.0	60.2	1.51	17.71	0.6732	7.09
" 31	10.65	44.4	200	244.0	56.4	1.42	16.29	0.6713	7.06
" 31	10.65	44.4	300	344.0	49.3	1.21	16.18	0.6739	7.10
" 31	10.65	44.4	500	544.0	48.9	1.14	16.72	0.6732	7.09
" 31	10.65	44.4	700	744.0	47.5	1.04	16.96	0.6715	7.06
1926									
Apr. 30	9.62	51.8	0	51.8	64.3	1.74	26.91	0.6862	7.29
" 30	9.62	51.8	35	87.0	58.5	1.58	26.32	0.6883	7.32
" 30	9.62	51.8	90	142.0	47.9	1.27	25.00	0.6913	7.37
" 30	9.62	51.8	165	217.0	48.4	1.26	25.14	0.6892	7.34
June 7	10.84	52.9	0	52.9	23.3	0.615	21.83	0.7123	7.71
" 8	10.84	52.9	0	52.9	54.5	1.44	28.91	0.6950	7.43
" 10	11.09	49.7	0	49.7	41.2	1.08	24.88	0.6976	7.51

TABLE IV (*Fig. 4*).

$pK_1'_{(H_2CO_3)}$ in 15 mM Carbon Monoxide Hemoglobin Solution at Varying Ionic Strength.

Constants.

$t = 38°$

$e_0 = 0.2366$ volt.

Date.	Total Hb.	Available base.	NaCl	Γ	CO_2 tension.	H_2CO_3	$NaHCO_3$	E.M.F. corrected.	pa_H
1926	*mM per l.*	*mM per l.*	*mM per l.*	*mM per l.*	*mm. Hg*	*mM per l.*	*mm per l.*	*volt*	
May 10	15.78	60.5	0	60.5	65.8	1.55	24.19	0.6864	7.29
" 10	15.78	60.5	60	121.0	64.5	1.50	24.19	0.6850	7.27
" 10	15.78	60.5	150	211.0	58.4	1.32	24.04	0.6855	7.28

pK_1' in Solutions of RHb at Varying Ionic Strength.—The data (see Tables V, VI, and VII and Figs. 5, 6, and 7) are of a similar

TABLE V (*Fig. 5*).

$pK_1'_{(H_2CO_3)}$ in 5 mM Reduced Hemoglobin Solution at Varying Ionic Strength.

Constants.

$t = 38°$

$e_0 = 0.2366$ volt.

Date.	Total Hb.	Available base.	NaCl	Γ	CO_2 tension.	H_2CO_3	$NaHCO_3$	E.M.F. corrected.	p_H
1926	*mM per l.*	*mM per l.*	*mM per l.*	*mM per l.*	*mm. Hg*	*mM per l.*	*mM per l.*	*volt*	
May 14	5.72	29.8	0.0	29.8	40.3	1.18	17.33	0.6866	7.29
" 14	4.96	25.8	69.3	95.0	46.1	1.35	16.72	0.6789	7.17
" 14	5.72	29.8	170.0	200.0	43.9	1.23	18.59	0.6812	7.21
" 20	4.49	22.5	0.0	22.5	36.4	1.09	12.78	0.6840	7.25
" 20	4.49	22.5	0.0	22.5	39.4	1.18	13.14	0.6800	7.19
" 20	4.49	22.5	85.0	108.0	30.1	0.89	12.23	0.6829	7.24
" 20	4.49	22.5	185.0	208.0	31.9	0.92	12.83	0.6796	7.18
June 10	5.92	18.0	0.0	18.0	28.2	0.83	11.02	0.6839	7.25
" 10	5.92	18.0	0.0	18.0	28.9	0.85	11.29	0.6836	7.25

TABLE VI (*Fig. 6*).

$pK_1'_{(H_2CO_3)}$ in 10 mM Reduced Hemoglobin Solution at Varying Ionic Strength.

Constants.

$t = 38°$

$e_0 = 0.2366$ volt.

Date.	Total Hb.	Available base.	NaCl	Γ	CO_2 tension.	H_2CO_3	$NaHCO_3$	E.M.F. corrected.	p_H
1926	*mM per l.*	*mM per l.*	*mM per l.*	*mM per l.*	*mm. Hg*	*mM per l.*	*mM per l.*	*volt*	
Nov. 10	10.86	36.5	0	36.5	43.4	1.15	15.07	0.6781	7.17
" 10	10.86	36.5	25	62.0	43.7	1.15	15.21	0.6773	7.15
" 10	10.86	36.5	50	87.0	38.4	1.00	14.79	0.6782	7.17
" 10	10.86	36.5	100	137.0	45.1	1.16	16.24	0.6757	7.13
" 10	10.86	36.5	150	187.0	44.4	1.13	15.62	0.6744	7.11
1926									
June 10	11.09	33.7	0	33.7	68.8	1.83	22.03	0.6768	7.14
" 10	11.09	33.7	0	33.7	67.2	1.79	22.21	0.6782	7.16

nature on reduced hemoglobin in concentrations of 5 and 10 and 13 mM over an ionic strength range of 10 to 200 mM. The points

also fall approximately on straight lines again, indicating a relation of the Debye-Hückel form. The equations for the lines are

RHb per liter. mM		
5	$pK_1' = 6.21 - 0.42\sqrt{\Gamma}$	(21)
	$\log \gamma_* = -0.12$	(22)
10	$pK_1' = 6.14 - 0.34\sqrt{\Gamma}$	(23)
	$\log \gamma_* = -0.19$	(24)
13	$pK_1' = 6.06 - 0.23\sqrt{\Gamma}$	(25)
	$\log \gamma_* = -0.27$	(26)

TABLE VII (*Fig. 7*).

$pK_1'_{(H_2CO_3)}$ in 13 mM Reduced Hemoglobin at Varying Ionic Strength.

Constants.

$t = 38°$
$e_0 = 0.2350$ volt.
Hb = 12.91 mM per liter.
Available base = 43.2 mM per liter.

Date.	NaCl	Γ	CO_2 tension.	H_2CO_3	$BHCO_3$	E.M.F. corrected to 760 mm. H_2.	pa_H
1927	*mM per l.*	*mM per l.*	*mm. Hg*	*mM per l.*	*mM per l.*	*volt*	
Feb. 16	0	43.2	109.6	2.78	30.96	0.6711	7.07
" 18	0	43.2	85.9	2.17	29.50	0.6751	7.14
" 17	51.4	94.6	63.2	1.57	27.02	0.6795	7.21
" 17	119.4	162.6	78.6	1.93	28.48	0.6763	7.15
" 17	217.0	260.2	85.2	2.04	29.34	0.6734	7.10
" 18	230.0	273.2	82.8	1.97	29.18	0.6744	7.12

pK_1' in Solutions of Methemoglobin at Varying Ionic Strength.—The crystallized methemoglobin was prepared from horse blood by electrodialysis, and solutions were prepared and equilibrated as with hemoglobin. The data are given in Table VIII and summarized in Fig. 8 where pK_1' is plotted against $\sqrt{\Gamma}$. In methemoglobin solutions it is difficult to get accurate pa_H values, but the results may be represented with fair accuracy by a line whose equation is

$$pK_1' = 6.08 - 0.28\sqrt{\Gamma} \quad (27)$$

$$\log \gamma_* = -0.25 \quad (28)$$

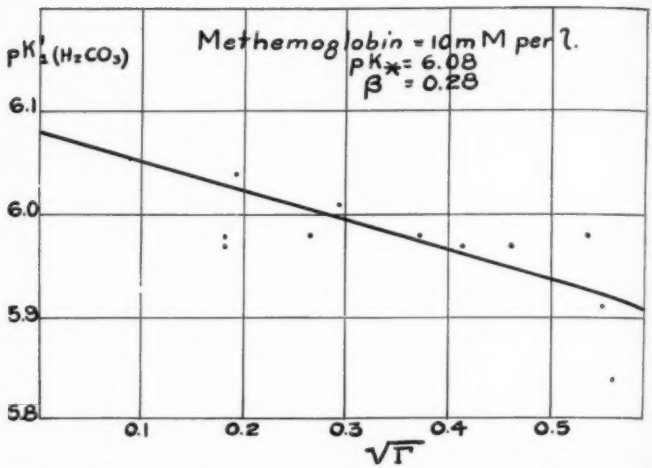

FIG. 8. $pK_1'_{(H_2CO_3)}$ in solutions of 10 mM methemoglobin at varying ionic strength.

TABLE VIII (*Fig. 8*).

$pK_1'_{(H_2CO_3)}$ in Solutions of 10 mM Methemoglobin at Varying Ionic Strength.

Constants. July 6, 1926.
$t = 38°$
$e_0 = 0.2366$ volt.

MHb	Available base.	NaCl	Γ	CO_2 tension.	Total CO_2.	H_2CO_3	E.M.F. corrected.	p_H
mM per l.	*mM per l.*	*mM per l.*	*mM per l.*	*mm. Hg*	*mM per l.*	*mM per l.*	*volt*	
10.84	70.2	0.0	70.2	78.0	24.60	2.04	0.6697	7.02
10.84	70.2	100.0	170.2	71.3	24.10	1.83	0.6719	7.06
10.84	70.2	250.0	320.2	70.8	25.25	1.74	0.6674	6.98
9.27	37.4	0.0	37.4	62.2	18.28	1.70	0.6702	7.03
9.27	37.4	250.0	287.4	66.4	21.42	1.71	0.6714	7.04
10.25	32.2	0.0	32.2	39.1	14.02	1.05	0.6721	7.06
10.25	32.2	0.0	32.2	49.7	16.59	1.33	0.6711	7.05
10.25	32.2	55.0	87.2	51.1	17.19	1.35	0.6737	7.08
10.25	32.2	100.0	132.2	44.7	16.56	1.17	0.6746	7.10
10.25	32.2	180.0	212.2	48.0	17.91	1.23	0.6753	7.11
10.25	32.2	270.0	302.2	57.9	20.31	1.45	0.6698	7.02

pK_1' of Carbonic Acid in Solutions of Cyanhemoglobin.—The solutions were prepared from crystallized cyanhemoglobin. The pK_1' values were determined at a 10 mM concentration of HbCN and varying ionic strengths. The data are given in Table IX

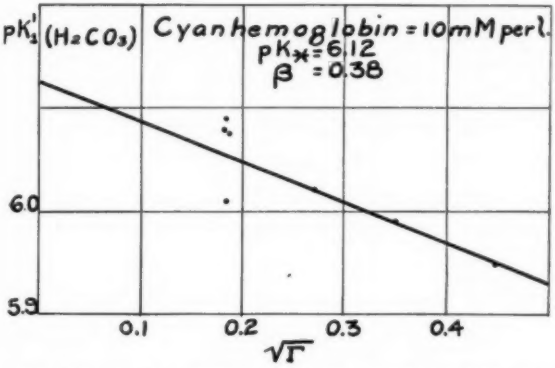

FIG. 9. $pK_1'_{(H_2CO_3)}$ in solutions of 10 mM cyanhemoglobin at varying ionic strength.

TABLE IX (*Fig. 9*).

$pK_1'_{(H_2CO_3)}$ in Solutions of 10 mM Cyanhemoglobin at Varying Ionic Strength.

Constants. Oct. 7, 1926.
HbCN = 9.57 mM per liter.
Available base = 33.3 mM per liter.
t = 38°
e_0 = 0.2367 volt.

NaCl	Γ	CO_2 tension.	Total CO_2.	H_2CO_3	E.M.F. corrected.	ps_H
mM per l.	*mM per l.*	*mm. Hg*	*mM per l.*	*mM per l.*	*volt*	
0	33	61.1	21.02	1.66	0.6738	7.08
40	73	60.1	20.55	1.62	0.6737	7.08
90	123	57.6	22.14	1.53	0.6757	7.12
170	203	56.6	22.46	1.48	0.6743	7.10

and summarized in Fig. 9. Again the straight line relation of the limiting law of Debye and Hückel between pK_1 and $\sqrt{\Gamma}$ is apparent and we have

$$pK_1' = 6.12 - 0.38\sqrt{\Gamma} \quad (29)$$

$$\log \gamma_* = -0.21 \quad (30)$$

pK_1' of Carbonic Acid in Solutions of Nitric Oxide Hemoglobin.—The solution of HbNO was prepared in such a way as practically to eliminate

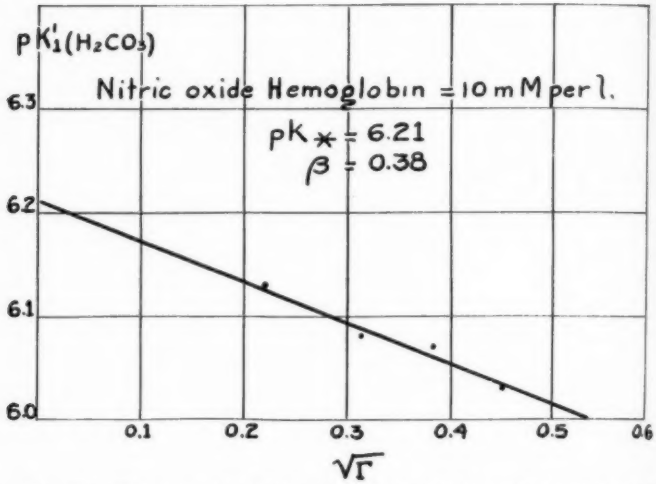

FIG. 10. $pK_1'_{(H_2CO_3)}$ in solutions of 10 mM nitric oxide hemoglobin at varying ionic strength.

TABLE X (*Fig. 10*).

$pK_1'_{(H_2CO_3)}$ in Solutions of 10 mM Nitric Oxide Hemoglobin at Varying Ionic Strength.

Constants. Nov. 18, 1926.
HbNO = 10.01 mM per liter.
Available base = 49.4 mM per liter.
t = 38°
e_0 = 0.2353 volt.

NaCl	Γ	CO_2 tension.	Total CO_2.	H_2CO_3	E.M.F. corrected.	p_H
mM per l.	*mM per l.*	*mm. Hg*	*mM per l.*	*mM per l.*	*volt*	
0	49.4	94.2	28.48	2.52	0.6758	7.14
50	99.4	112.9	30.43	2.99	0.6695	7.04
100	149.4	116.1	31.45	3.04	0.6695	7.04
150	199.4	106.6	31.56	2.75	0.6702	7.05

the possibility of nitric acid formation. This was accomplished by excluding oxygen at all steps. A solution of hemoglobin was prepared from the

crystals in the usual way and thoroughly reduced by repeated saturations with hydrogen and a high (150 mm. of Hg) CO_2 pressure. NO was prepared from concentrated HNO_3 and copper was washed by prolonged rotation in a liter tonometer with strong NaOH. The two tonometers were connected to the gas manifold which was then washed out with hydrogen. The nitric oxide was then transferred to the tonometer containing the reduced hemoglobin solution which was then rotated for ½ to 1 hour. This was repeated once or twice and the solution was then separated without contact with air. No gross changes occurred in the solution except a change in color to dull ruby, and after separation from gaseous NO the solution could be handled in air without any apparent change. The characteristic spectrum of HbNO was found and the oxygen capacity was zero.

TABLE XI (*Fig. 11*).

pK_* (H_2CO_3) in Solutions of Carbon Monoxide and Reduced Hemoglobin at Concentrations of 2.5 to 19 mM per liter.

Constants. June 28, 1926.
$t = 38°$
$e_0 = 0.2366$ volt.
NaCl = 0

State.	Hb	Γ	CO_2 tension.	Total CO_2.	H_2CO_3	E.M.F. corrected.	pa_H	pK_1'
	mM per l.	*mM per l.*	*mm. Hg*	*mM per l.*	*mM per l.*	*volt*		
HbCO.....	15.96	82.2	111.5	46.23	2.61	0.6830	7.24	6.02
"	9.81	41.2	56.3	20.60	1.52	0.6814	7.21	6.11
"	5.28	22.2	26.5	10.93	0.784	0.6866	7.29	6.18
"	2.53	10.6	13.6	5.46	0.424	0.6851	7.27	6.19
RHb.....	18.86	79.3	119.6	48.00	2.61	0.6796	7.18	5.94
"	9.81	41.2	64.2	26.45	1.74	0.6805	7.20	6.04
"	5.28	22.2	31.3	14.14	0.927	0.6850	7.27	6.11
"	2.53	10.6	19.0	7.10	0.591	0.6834	7.25	6.21

The pK_1' values in a 10 mM solution of HbNO were determined as in the other solutions. Table X and Fig. 10 show the data which again yield a straight line relation.

$$pK_1' = 6.21 - 0.38\sqrt{\Gamma} \quad (31)$$

$$\log \gamma_* = -0.12 \quad (32)$$

pK_* in HbCO and RHb in One Sample of Hemoglobin.—The pK_* and $\log \gamma_*$ at varying hemoglobin concentrations given above were

obtained mostly from points on many samples of hemoglobin and over a long time interval. While the agreement is good, we sought to determine more exactly the relation between pK_* and hemoglobin concentration by using a solution prepared from one batch of hemoglobin crystals. We therefore determined pK_1' in the presence of HbCO and RHb respectively at concentrations of 2.5, 5, 10, 15, and 19 mM. The protocols are given in Table XI,

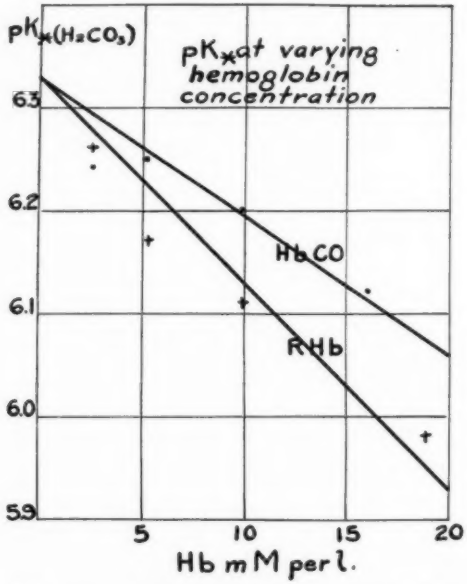

FIG. 11. pK_* (H_2CO_3) in reduced and carbon monoxide hemoglobin at 2.5, 5, 10, 15, and 19 mM per liter.

and in Fig. 11 we have plotted the data. It is obvious that, since by dilution the ionic strengths vary from 80 to 10 mM per liter, it is necessary to calculate pK_* by extrapolating the experimental pK_1' values to $\Gamma = 0$ by use of the equation $pK_* = pK_1 + \beta\sqrt{\Gamma}$ and the previously observed β values for each hemoglobin concentration given in Fig. 13. In the case of HbCO, if we ignore the point at HbCO = 2.5 mM the representative straight line through

the three remaining points extrapolated to Hb = 0 gives $pK_* = pK_1 = 6.32$ in excellent agreement with 6.33, the value in aqueous solution. Disregarding the slight difference we have for HbCO

$$pK_* = 6.33 - 0.014\,[Hb] \tag{33}$$

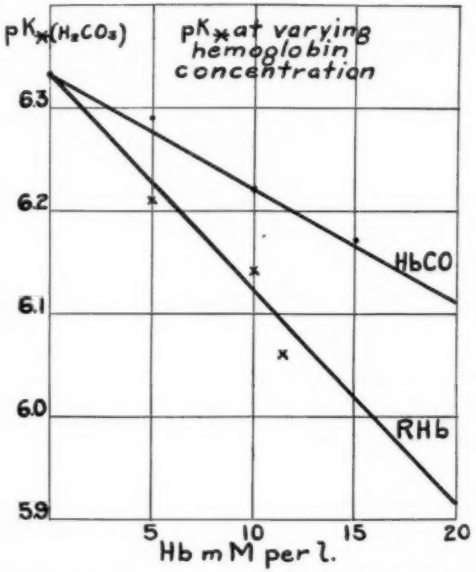

FIG. 12. Experimental $pK_{*(H_2CO_3)}$ values at varying hemoglobin concentrations.

from which

$$\log \gamma_* = -0.014\,[Hb] \tag{34}$$

For RHb a line through the experimental points would show some curvature which would also approximately extrapolate to $pK_* = pK_1 = 6.33$. Whether this curvature is valid or significant we do not know; therefore we have chosen to ignore it and have drawn a straight line through the points whose equation is

$$pK_* = 6.33 - 0.020\,[RHb] \tag{35}$$

from which

$$\log \gamma_* = -0.020\ [\mathrm{RHb}] \tag{36}$$

Variation of pK_*, log γ_*, and β with Hemoglobin Concentration.— In Table XII are collected all the data (except those given in Table XI) on the calculated pK_*, log γ_*, and β values. In Fig. 12 pK_* is plotted against Hb concentration. For both HbCO and

TABLE XII (*Figs. 12 and 13*).

Experimental pK_*; Log γ_*, and β Values at Varying Hemoglobin Concentrations.

Concentration of Hb.	MtHb			HbCN			HbNO		
	pK_*	Log γ_*	β	pK_*	Log γ_*	β	pK_*	Log γ	β
mM per l.									
0	6.33	0.00	0.54	6.33	0.00	0.54	6.33	0.00	0.54
10	6.08	0.25	0.28	6.12	0.21	0.38	6.22	0.11	0.38

TABLE XIII.

Constants for the Calculation of pK_1' and $\gamma_{HCO_3^-}$ in Hemoglobin Solutions from Hb = 0 to 20 mM per liter and Γ = 0 to 300 mM per liter.

Hb derivative.	ρ	σ
HbCO	0.012	0.0115
HbNO	0.012	0.0160
RHb	0.021	0.0214
MtHb	0.025	0.0260
HbCN	0.021	0.0160

RHb a straight line may be drawn through 6.33, approximating the other points with a deviation of $\pm$ 0.02; *i.e.*,

$$pK_* = 6.32 - \rho\ [\mathrm{Hb}] \tag{37}$$

For HbCO and RHb there are three points obtained from the composite data and the values of ρ = 0.011 and 0.021 respectively agree quite well with the values 0.014 and 0.020 obtained from pK_* on a single sample of hemoglobin (Equations 33 and 34). In Fig. 13 β is plotted against [Hb]. The variations are greater than in the case of pK_*. Nevertheless we may draw straight lines from

0.54, the value in water, through the points with fair approximation. Then

$$\beta = 0.54 - \sigma\,[\mathrm{Hb}] \tag{38}$$

Including $\beta_0 = 0.54$ in water we have four points each for HbCO and RHb. For MtHb, HbCN, and HbNO there are only two points each. We have calculated σ and likewise ρ for all the derivatives in Table XIII. These constants measure the effect of

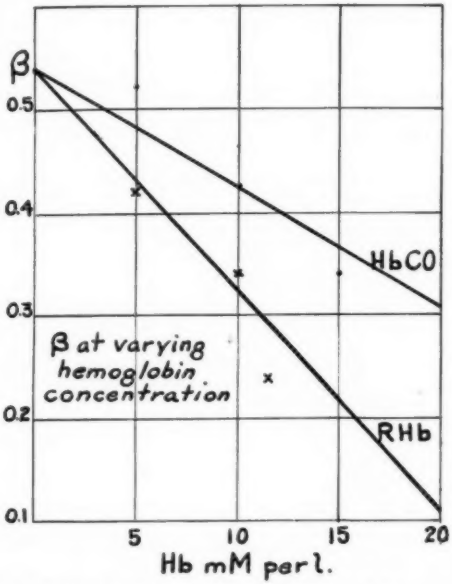

FIG. 13. Experimental β values at varying hemoglobin concentrations.

the hemoglobin derivatives on pK_1'; *i.e.*, on log $\gamma_{HCO_3^-}$. On the basis of ρ the pigments fall sharply into two groups: (1) HbCO and HbNO $\rho = 0.012$ to 0.013 and (2) RHb, MtHb, and HbCN $\rho = 0.021$ to 0.025. This division on the basis of σ is not so sharp but nevertheless quite apparent. In other words pK_1' in hemoglobin solutions varies with each derivative. In Paper V we shall indicate a possible physical significance of these constants.

Calculation of pK_1' at All Concentrations of Hemoglobin and Γ.—Using Equations 37 and 38 and the equation

$$pK_1' = pK_* - \beta\sqrt{\Gamma} \tag{39}$$

we may closely approximate pK_1' in the range of Hb, 0 to 20 mM per liter and Γ = 0 to 500 mM per liter in various hemoglobin derivatives (RHb, HbCO, MtHb, HbCN, HbNO). The constants ρ and σ are given in Table XIII. The activity coefficients $\gamma_{HCO_3^-}$, γ_*, γ_0 may likewise be calculated from the constants and the equations

$$\log \gamma_{HCO_3^-} = pK_1' - pK_1 \tag{8}$$

From Equations 4 and 37

$$\log \gamma_* = pK_* - pK_1 \tag{4}$$

$$= -\rho\,[Hb] \tag{40}$$

From Equations 4, 5, 8, and 38

$$\log \gamma_0 = \log \gamma_{HCO_3^-} - \log \gamma_* \tag{5}$$

$$= pK_1 - pK_*$$

$$= -\beta\sqrt{\Gamma}$$

$$= -[\beta_0 - \sigma\,[Hb]] \tag{41}$$

We have plotted in Fig. 14 the values of pK_1' against $\sqrt{\Gamma}$ for reduced and carbon monoxide hemoglobin at concentrations of 10 and 20 mM. The graphs bring out clearly the effects of hemoglobin concentration and ionic strength as well as the difference between HbCO and RHb on these factors.

pK_1' of Carbonic Acid within the Red Blood Cell.—In order to assign a value to pK_1' in the hemoglobin solution within the red blood cell we must make two assumptions, purely tentative. First that the physical state of hemoglobin in solution within the cell is not so different from that in our experimental solutions as to influence markedly the dissociation of carbonic acid or the activity of the bicarbonate ion. The preponderance of hemoglobin over

other cell constituents is in favor of this. Second that the pK_1' determined for carbon monoxide hemoglobin is the same as for oxyhemoglobin. This is admittedly a more tenuous assumption but we cite the chemical similarity of these two forms of hemoglobin in their isoelectric points (O_2 or CO), dissociation curves, and CO_2 absorption curves, particularly the identical increase in

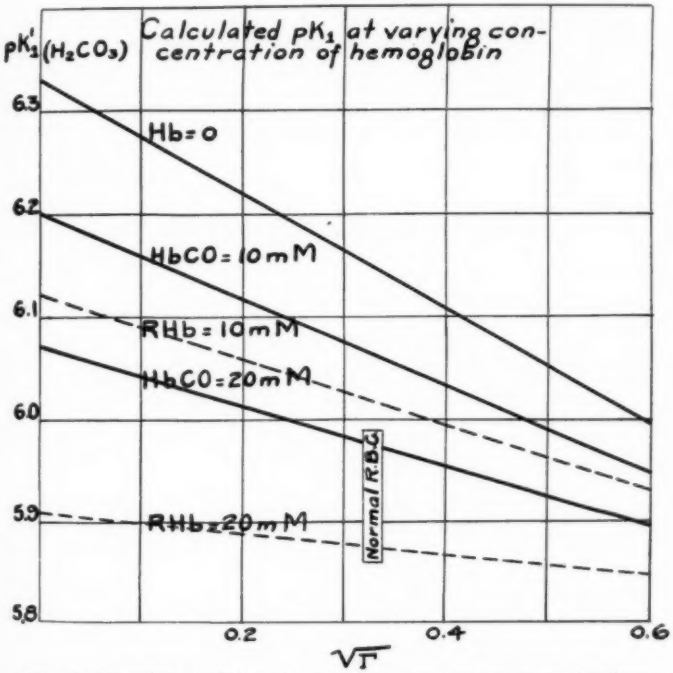

FIG. 14. Calculated $pK_1'_{(H_2CO_3)}$ in reduced and carbon monoxide hemoglobin at 10 and 20 mM per liter.

base bound at constant pH in passing from reduced hemoglobin to HbCO or HbO_2, as lending it some support.

The concentration of hemoglobin within the normal human red blood cell is quite constant and is approximately 20 mM per liter. The ionic strength is also quite constant at $\Gamma = 100$ to 120 mM per liter of cells. For oxyhemoglobin then pK_1' is 5.98 and for re-

duced hemoglobin it is 5.87; this latter value is in approximate agreement with the value of 5.93 for laked horse red blood cells obtained by Van Slyke, Hastings, Murray, and Sendroy (1925).

Our experiments on single samples of hemoglobin solutions have consistently shown pK_1' to be higher in the presence of carbon monoxide hemoglobin than in reduced hemoglobin. At the hemoglobin and ionic concentration of the red blood cell the difference is small, it is true, and while we are reasonably confident that *in vitro* the $pK_1'_{(H_2CO_3)}$ in the presence of reduced hemoglobin is less by 0.1 than in the presence of carbon monoxide hemoglobin (and by assumption oxyhemoglobin), we cannot be positive of the reality or physiological significance of this difference within the interior of the red blood cell.

TABLE XIV.

$pK_1'_{(H_2CO_3)}$ in Hemolyzed Horse Red Blood Cells.

Constants. Oct. 25, 1926.
t = 38°
Hb = 18.22 mM per liter.
Cl = 103 m.-eq. per liter.
Dry weight = 38.1 per cent.
e_0 = 0.2367 volt.

State.	Γ	CO_2 tension.	H_2CO_3	Total CO_2.	E.M.F. corrected.	pa_H	pK_1' observed.	pK_1' calculated.
	mM per l.	*mm. Hg*	*mM per l.*	*mM per l.*	*volt*			
HbCO	238	120.4	2.24	35.98	0.6751	7.10	5.92	5.94
RHb	177	72.3	1.37	19.30	0.6669	6.97	5.85	5.89

We have sought to test this point more directly by determining pK_1' on hemoglobin solutions obtained from cell emulsions by hemolysis. Horse red blood cells were washed three times with a 30 mM $NaHCO_3$, 150 mM NaCl solution adjusted to a pH of about 7.40. The heavily packed cells were hemolyzed by saponin. The supernatant fluid was carefully examined to be certain no cell forms were present. The equilibration of the reduced and carbon monoxide hemoglobin was done in the usual way and the pa_H, base, chloride, hemoglobin, and carbonates were determined. Analyses were made of weighed samples and the volumes calculated from the observed specific gravity. This is essential since

a concentrated saponized solution of red blood cells is so viscous that accurate pipetting is impossible. The results are given in Table XIV.

It was found that the conversion of cell contents after hemolysis into carbon monoxide hemoglobin resulted in an abundant crystal-

TABLE XV.

$pK_1'_{(H_2CO_3)}$ in Hemolyzed Beef and Human Red Blood Cells.

Constants.

A. Beef cells, Nov. 17, 1926.

Hb = 15.81 mM per liter.
Total base = Γ = 121.1 m.-eq. per liter.
Cl = 56.6 m.-eq. per liter.
e_0 = 0.2353 volt.
Dry weight = 0.2775 kilo per liter.

B. Human cells, Nov. 26, 1926.

Hg = 20.02 mM per liter.
Total base = Γ = 142.2 m.-eq. per liter.
Cl = 57.3 m.-eq. per liter.
α_{CO_2} = 0.0208
e_0 = 0.2353 volt.
Dry weight = 0.3805 kilo per liter.

State.	CO_2 tension.	H_2CO_3	Total CO_2.	E.M.F. corrected.	p_H	pK_1' observed.	Mean.	pK_1' calculated.
	mm. Hg	*mM per l.*	*mM per l.*	*volt*				
A. HbCO	78.9	1.85	18.16	0.6647	6.96	6.01	6.02	6.02
"	80.8	1.90	18.35	0.6651	6.97	6.03		
RHb	82.1	1.93	22.70	0.6673	7.00	5.97	5.97	5.93
"	85.3	2.00	23.70	0.6682	7.02	5.98		
						Difference.	0.05	0.09
B. HbCO	55.4	1.15	13.1	0.6686	7.03	6.01	6.02	5.96
"	47.2	0.98	11.7	0.6693	7.06	6.02		
RHb	33.5	0.68	13.1	0.6785	7.09	5.94	5.94	5.87
						Difference.	0.08	0.09

lization. To avoid this we were compelled to add an excess of base to that sample. However, the pK_1' values observed agree quite well with the calculated values from Fig. 12 and the pK_1' difference is again apparent.

We were anxious to obtain a further confirmation of the pK_1' difference in cell contents as little changed as possible; we there-

fore resorted to beef blood which we centrifuged at high speed in small tubes for 1 hour to effect complete separation of cells. The mixed cell magna was diluted with 4 volumes of water to effect hemolysis, centrifuged, and the supernatant liquid concentrated by evaporation at 38° to its original volume, yielding a solution of low viscosity and free of cells as shown by careful microscopic examination. As before, analyses were made of weighed samples, the volumes being calculated from the observed specific gravity. Table XV contains the data. The pK_1' values are in good agreement with those calculated from ρ and σ, and as

TABLE XVI.

$pK_1'_{(H_2CO_3)}$ in Reduced and Carbon Monoxide Hemoglobin with Excess of Base.

Constants. Oct. 19, 1926.
Hb = 9.92 mM per liter.
t = 38°
Γ = base.
e_0 = 0.2367 volt.
NaCl = 0

State.	Available base.	CO_2 tension.	Total CO_2.	H_2CO_3	E.M.F. corrected.	pa_H	pK_1' observed.	pK_1' calculated.
	mM per l.	*mm. Hg*	*mM per l.*	*mM per l.*	*volt*			
HbCO	31.3	61.6	16.09	1.67	0.6741	7.08	6.14	6.12
RHb	31.3	62.7	21.15	1.69	0.6735	7.07	6.01	6.07
HbCO	60.0	103.3	41.85	2.78	0.6850	7.27	6.12	6.10
"	60.0	127.2	45.41	3.42	0.6816	7.21	6.12	6.10
RHb	60.0	131.9	49.84	3.53	0.6788	7.17	6.05	6.04
"	60.0	129.0	48.50	3.47	0.6784	7.16	6.05	6.04

before the difference of the pK_1' values in HbCO and RHb is apparent. The experiment was repeated on human blood cells, hemolysis being brought about by saponin. Analyses were again made of weighed samples. The results (Table XV) are in conformity with those on horse and beef cells. While the pK_1' values found are higher than calculated, the difference in pK_1' of 0.08 is in agreement with the calculated difference of 0.09.

pK_1' in Reduced and Carbon Monoxide Hemoglobin in Presence of Excess of Base.—The relative insolubility of carbon monoxide hemoglobin may lead to crystallization from solutions if the avail-

able base fall below a certain minimum. We observed that such crystallization occurred in some of our solutions after long standing at room temperatures, although it was never apparent during the course of equilibration or pa_H determination. Nevertheless we thought it possible that the difference of the pK_1' values between RHb and HbCO might be due to a partial crystallization of HbCO, giving a dilute HbCO solution and hence a higher pK_1'. We tested this by pK_1' determinations in RHb and HbCO solutions, adding such an excess (twice that needed for solutions of the HbCO) of NaOH that crystallization was undoubtedly excluded.

TABLE XVII.

$pK_1'_{(H_2CO_3)}$ in Aqueous Solution at Constant Ionic Strength but Varying pa_H.

Constants. Feb. 17, 1926.
$t = 38°$
$e_0 = 0.2360$ volt.

$NaHCO_3$	NaCl	Γ	P_{CO_2} tension.	H_2CO_3	E.M.F. corrected.	pa_H	pK_1'
mM per l.	*mM per l.*	*mM per l.*	*mm. Hg*	*mM per l.*	*volt*		
20.0	0	20.0	620	20.20	0.6130	6.11	**6.11**
19.33	0	19.3	611	19.9	0.6216	6.24	6.25
20.0	0	20.0	307	9.98	0.6369	6.50	6.20
20.0	0	20.0	304	9.88	0.6280	6.32	6.21
19.41	0	19.4	69.7	2.26	0.6797	7.18	6.24
						Calculated.	**6.25**

The data (Table XVI) showed that pK_1' was the same with high or low base, the results being in agreement with our calculated values and showing that a diminution of the HbCO in solutions by crystallization did not occur and was not a source of error in the pK_1' measurements.

Constancy of $pK_1'_{(H_2CO_3)}$ at Constant Ionic Strength but Varying pa_H and the Activity Coefficient of the Carbonate Ion of Hemoglobin Bicarbonate. (*a*) *In Simple Aqueous Solution.*—The assumption that pK_1' of carbonic acid in $NaHCO_3$-H_2CO_3-NaCl systems is the same regardless of the pa_H, is a natural one and is repeatedly made without, so far as we know, experimental proof. We have tested it in simple aqueous solutions, using a solution of 20 mM $NaHCO_3$. The data (except for the first) (Table XVII) indicate that pK_1' is

TABLE XVIII.

$pK_1'_{(H_2CO_3)}$ in Carbon Monoxide Hemoglobin Solution at Constant Ionic Strength but Varying pa_H.

Constants. May 20, 1926.
HbCO = 8.09 mM per liter.
Available base = 57.2 mM per liter.
NaCl = 0
t = 38°
e_0 = 0.2366 volt.
Γ = 57.2 mM per liter.

CO_2 tension.	Total CO_2.	H_2CO_3	E.M.F. corrected.	pa_H	pK_1'
mm. Hg	*mM per l.*	*mM per l.*	*volt*		
21.4	23.60	0.595	0.7114	7.70	6.11
46.7	28.81	1.298	0.6961	7.45	6.12
154.9	40.65	4.305	0.6670	7.05	6.12
227.5	45.86	6.32	0.6557	6.89	6.09
253.5	46.83	7.05	0.6495	6.80	6.05

TABLE XIX.

$pK_1'_{(H_2CO_3)}$ in Reduced Hemoglobin Solution at Constant Ionic Strength but Varying pa_H.

Constants. Nov. 2, 1926.
RHb = 10.6 mM per liter.
e_0 = 0.2367 volt.
Γ = 133 mM per liter.
pK_1' = 5.99 calculated.

Available base.	NaCl	CO_2 tension.	Total CO_2.	H_2CO_3	HCO_3^-	$\frac{HbHCO_3}{Total\ HCO_3}$	E.M.F. corrected.	pa_H	pK_1'
mM per l.	*mM per l.*	*mm. Hg*	*mM per l.*	*mM per l.*	*mM per l.*	*per cent*	*volt*		
11.5	121	12.9	5.1	0.334	4.8	0	0.6727	7.07	5.92
14.2	118	26.4	29.3	6.87	22.4	36	0.6386	6.51	6.00
11.5	121	29.1	28.6	7.54	21.1	46	0.6335	6.43	5.98
4.8	128	28.4	25.7	7.38	18.3	74	0.6307	6.39	6.00
−6.0*	133	29.0	11.1	7.55	4.5	100	0.5832	5.64	5.96
								Mean.....	5.97 ± 0.026

* Excess of HCl over total available base.

constant over the wide pH range studied (6.2 to 7.2), the values being in agreement with our previous results.

(*b*) *In Solutions of Hemoglobin.*—We have just shown that $pK_1'_{(H_2CO_3)}$ is constant within wide limits of pa_H. Whether this constancy exists in hemoglobin solutions is of practical and theoretical significance. We have tested the question experimentally in two samples of hemoglobin, the data being shown in Tables XVIII and XIX. In the first sample equilibrated as HbCO the available base was relatively high; therefore the pa_H change secured by variation of the CO_2 pressure was comparatively limited, ranging from 6.8 to 7.7. The pK_1' is quite constant to pH 7.05, below which there is a decrease of 0.07.

We repeated the experiment on a second sample of hemoglobin. This was first converted into reduced hemoglobin and part of the available base was then neutralized by HCl. In this way we were able to get pH values as low as 5.6 without apparent change in the hemoglobin. Except for the first determination all of the pH values are below the isoelectric point (6.8) of hemoglobin. Within a pH range of 5.64 to 7.07 pK_1' is practically constant (5.97 ± 0.026) in good agreement with the value 5.99, calculated from ρ and σ.

It will be noted that the concentration of HCO_3^- except at pH 7.07 exceeds the concentration of available Na. In other words part of the HCO_3^- ion was combined with hemoglobin as bicarbonate as shown in the seventh column of Table XIX. Since pK_1' is here found to be constant, we find by Equation 8 that $\gamma_{HCO_3^-}$ is constant. This is consistent with our previous assumptions that $HbHCO_3$ is completely dissociated and that in hemoglobin solutions at the same ionic strength the activity coefficient of the bicarbonate ion is constant whether the associated cation is Na or hemoglobin.

SUMMARY AND CONCLUSIONS.

1. The apparent first dissociation constant, pK_1', of carbonic acid was calculated from the electrometric pa_H, and stoichiometrical total CO_2 and P_{CO_2} in solutions of varying concentrations of carbon monoxide hemoglobin, reduced hemoglobin, methemoglobin, cyanhemoglobin, and nitric oxide hemoglobin and varying

ionic strengths. These pK_1' values when plotted against the $\sqrt{\Gamma}$ give linear equations of the form, $pK_1' = pK_* - \beta\sqrt{\Gamma}$.

2. The total activity coefficient of the bicarbonate ion was calculated from pK_1'. In all cases log $\gamma_{HCO_3^-}$ plotted against $\sqrt{\Gamma}$ gave a straight line corresponding to the linear relation, log $\gamma_{HCO_3^-} = -\beta\sqrt{\Gamma}$, of the limiting equation of the Debye-Hückel theory. When Hb = 0, the theoretical $\beta_0 = 0.54$ is obtained. With increasing Hb concentration, however, β decreases, the extent of decrease varying with the hemoglobin derivative. β can be expressed by the equation $\beta = \beta_0 - \sigma$ [Hb].

3. pK_*, the value of pK_1' at finite hemoglobin concentrations and $\Gamma = 0$, also decreased according to the equation $pK_* = 6.33 - \rho$ [Hb]. ρ and σ are constants characteristic of the derivative of hemoglobin. On the basis of these constants the derivatives may be divided into two groups: (1) HbCO, HbNO; (2) RHb, MtHb, HbCN.

4. The activity coefficient $\gamma_{HCO_3^-}$ can be equated to two activity coefficients, γ_* and γ_0, and calculated by the equations

$$\log \gamma_{HCO_3^-} = \log \gamma_* + \log \gamma_0$$

$$\log \gamma_* = -\rho\,[\text{Hb}]$$

$$\log \gamma_0 = -[0.54 - \sigma\,[\text{Hb}]]\sqrt{\Gamma}$$

These activity coefficients allow the calculation of pK_1' over wide ranges of Hb and Γ. γ_* measures the effect of hemoglobin on $\gamma_{HCO_3^-}$, while γ_0 gives the effect of salt.

5. The calculated pK_1' in the red blood cell is tentatively calculated as 5.98 and 5.87 for the oxidized and reduced states respectively. These values were confirmed by direct determinations in hemolyzed human, horse, and beef cells, equilibrated as HbCO and RHb.

6. At constant Γ, pK_1 and $\gamma_{HCO_3^-}$ are constant in hemoglobin solutions at pa_H values above and below the isoelectric point, showing that the activity coefficients of HCO_3^- are the same whether combined with sodium or hemoglobin.

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STUDIES ON THE OXYGEN-, ACID-, AND BASE-COMBINING PROPERTIES OF BLOOD.

V. EXTENSION OF THE DEBYE-HÜCKEL THEORY OF IONIC INTERACTION TO HEMOGLOBIN, BICARBONATE-SODIUM CHLORIDE SYSTEMS.

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INTRODUCTION.

The Debye-Hückel theory of solutions was formulated in 1923. It has stimulated the reconsideration of a large amount of preexisting data and the projection of further experiments to test its

[1] A table of the symbols used will be found at the end of this paper.

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range of validity. Practically all of this work deals with simple aqueous solutions of one or at most two electrolytes, although there is a limited amount of data in such solutions as water-alcohol-salt and water-sugar-salt. The extension of the theory to biological systems in which there are water, a colloidal electrolyte (protein) in high weight concentration, many salts, and non-electrolytes, is beset with theoretical and practical difficulties. Nevertheless, the general validity of the theory has been so well established that this extension will undoubtedly prove profitable. For this reason we have considered the Hb, H^+, NaCl, HCO_3^-, CO_2 equilibria reported in the previous paper in the light of the Debye-Hückel theory and have attempted to determine the extent of conformity and non-conformity of the theory to our data.

We have discussed the subject always with reference to the particular system water, Hb, H^+, HCO_3^-, NaCl, CO_2. No pretence is made that the treatment is complete; much more experiment, specifically designed in the light of the theory, is needed before any extensive analysis of that behavior of water-protein-salt solutions can be given. In general, however, we may say that our data are satisfactorily in accord with the theory, but lack of direct or other indirect experimental data on the effect of hemoglobin on the dielectric constant of water makes it impossible at present to offer the agreement as proof. In other words, the Debye-Hückel theory of solutions offers us the only plausible explanation with which we are acquainted of the effect of hemoglobin on the activity of the bicarbonate ion.

Résumé of Experimental Data on $\gamma_{HCO_3^-}$ in Hemoglobin Solutions.—Let us for the moment disregard any theory as to the behavior of CO_2 in solutions containing $NaHCO_3$, NaCl, and hemoglobin or its derivatives, HbCO, MtHb, HbCN, HbNO. We have defined certain quantities, *viz.* pK_1, pK_1', pK_*, β_0, β, $\gamma_{HCO_3^-}$, γ_*, γ_0, and Γ, which may or may not have their usual theoretical significance. These values as defined by us are calculable from the experimentally determined CO_2 tension, total base, total CO_2, pa_H, chloride, and water content in a series of solutions whose hemoglobin content varied from 0 to 20 mM per liter. Our data clearly establish the following facts.

1. In water, as found by Hastings and Sendroy (1925), the relation pK_1, pK_1', β_0, and $\sqrt{\Gamma}$ is:

$$pK_1' = pK_1 - \beta_0 \sqrt{\Gamma} \quad (1)$$

$pK_1 = 6.33$ from which

$$\log_{HCO_3^-} = - \beta_0 \sqrt{\Gamma} \quad (2)$$

β_0 was found to be 0.54, practically the theoretical value.

2. In hemoglobin solutions we have found a similar relation for pK_1', pK_*, β, and $\sqrt{\Gamma}$:

$$pK_1 = pK_* - \beta \sqrt{\Gamma} \quad (3)$$

It was also found that

$$pK_* = pK_1 - \rho[Hb] \quad (4)$$

and

$$\beta = \beta_0 - \sigma[Hb] \quad (5)$$

ρ and σ are constants characteristic of the derivative of hemoglobin.

The activity coefficient $\gamma_{HCO_3^-}$ when divided into two coefficients, *i.e.*

$$\log \gamma_{HCO_3^-} = \log \gamma_* + \log \gamma_0 \quad (6)$$

could be calculated from the equations

$$\log \gamma_* = - \rho[Hb] \quad (7)$$

$$\log \gamma_0 = - [\beta_0 - \sigma[Hb]] \sqrt{\Gamma} \quad (8)$$

We will show that the Debye-Hückel theory of the behavior of electrolytes in solutions may be extended to concentrated hemoglobin solutions and that it offers a satisfactory explanation of these facts.

Debye-Hückel Theory in Aqueous Solutions.—The properties, *e.g.* vapor pressure, freezing point, boiling point, osmotic pressure, membrane equilibria, electromotive force phenomena, *etc.*, of very

dilute aqueous solutions of non-electrolytes and electrolytes can be quite satisfactorily explained by the application of the laws for ideal solution and the assumption that the (strong) electrolytes are completely ionized. In more concentrated solutions, however, the departure from the ideal state is great. Debye and Hückel (1923) explain in a satisfactory manner the behavior of strong electrolytes not only in water but in any solvent even in concentrated (3 to 5 M) solutions. Their theory evaluates the electrical effect of the charges of all the ions in the solution upon a given ion, A, in terms of the valence and concentration of all the ions, the valence, concentration, the effective ionic diameter of the given ion, and the dielectric constant of the solution.

The theory assumes nothing about ionization; it merely measures the effect on the properties of any given ion species of all ions present. Experiments on strong electrolytes show that as a rule they must be assumed to be completely ionized to account for this effect.

The salient features of the theory with emphasis on those aspects which relate to our problems will be discussed here. Since our discussion is limited to univalent ions (except in the case of the hypothetical hemoglobin ion discussed below), the Debye-Hückel equation may be written here

$$\log \gamma_i = - \frac{\beta_0 \sqrt{\Gamma}}{1 + Ba \sqrt{\Gamma}} + G \Gamma \qquad (9)$$

$\beta_0 = \dfrac{1.8 \times 10^6}{D_0^{\frac{3}{2}} T^{\frac{3}{2}}}$ (1.8×10^6 is calculated from universal constants.)

D_0 = dielectric constant of water.
T = absolute temperature.
β_0 = 0.505 at 25°.
β_0 = 0.532 at 38°.
B = $50.4a \times 10^8$ (50.4×10^8 is calculated from universal constants.)
$B_{25°}$ = $0.328a \times 10^8$.
$B_{38°}$ = $0.334a \times 10^8$.

G is a function which gives the variation of the dielectric constant of the solvent with Γ. $\Gamma = \frac{1}{2} \Sigma z^2[c]$; *i.e.* one-half the summation of the product of the concentration $[c]$ (in mols per liter) of each ion by its valence z squared, is the ionic concentration.

γ_i is the activity coefficient of the ion.

a = mean ionic collision sphere or distance in cm. of closest approach between the center of the ion A and the center of any surrounding positive or negative ion.

Relation of Γ *and* μ.—The equation

$$\Sigma \frac{\Gamma_i}{[c_i]} = \Sigma \frac{\mu_i}{(c_i)} \tag{10}$$

gives the relation between Γ the ionic strength per liter and μ the ionic strength per kilo of solvent. It is important to note that Equations 9 and 10 required that the ionic strength be expressed as mols per liter = Γ. Since

$$\Gamma = \tfrac{1}{2} \Sigma z^2 [c_i] = \tfrac{1}{2} [W] \Sigma z^2 (c_i) = [W] \mu$$

Equation 10 becomes

$$\log \gamma_i = -0.53 \sqrt{[W]}\, \mu \tag{11}$$

The use of Equation 11 without the $[W]$ factor leads to erroneous results in concentrated protein solutions; *e.g.*, in the red blood cell $[W] = 0.65$ and $\mu = 0.170$ mols per kilo of H_2O. If we assume hemoglobin to be without effect, $-\log \gamma_{HCO_3^-}$ is 0.176. By use of Equation 14 without the $[W]$ term, $-\log \gamma_{HCO_3^-} = 0.219$. The corresponding pK_1 values are 6.154 and 6.111. In serum $\mu = 0.160$ and $[W] = 0.9$ approximately. The above error is then only 0.010.

Valence of Hemoglobinate Ion in Calculation of Γ *and* μ.—In alkaline hemoglobin solutions a considerable part of the available base may exist as BHb; *i.e.*, we may have a fairly high concentration (10 to 40 mM per liter) of the colloidal ion $Hb^{-z\epsilon}$. There is enough evidence (Stadie and Martin, 1924) to show that 1 molecule of hemoglobin can bind 10 to 20 univalent base ions; *i.e.*, its valence is 10 to 20. Since $\Gamma = \frac{1}{2} \Sigma z^2 [c_i]$, the contribution of $Hb^{-z\epsilon}$ ion to Γ may be 100 to 400 times its concentration. The above value of z is approximated for a molecular weight of hemoglobin of 16,700, but z becomes 40 to 80 on the more probable value of $4 \times 16{,}700$ which would make the maximum Γ factor $\frac{6400}{4} = 1600$. Should we not multiply $[Hb^{-z\epsilon}]$ by some such factor

in calculating Γ? In view of the following consideration we believe this question should be answered negatively.

The introduction of z^2 into the ionic strength arises as follows: Call h_B the effect which charged ions of the A sort have upon given B ions. Using the Boltzman principle, Debye and Hückel derived an expression for the h_B which for simplicity we write

h_B = funct. ($n_A z_A \epsilon P_A$) where
n_A = number of A ions in unit volume.
z_A = valence of A.
ϵ = elementary electronic charge.
P_A = potential about B due to charges on A ions.

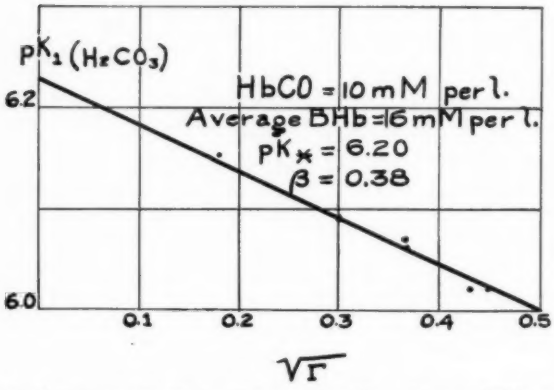

FIG. 1. Log $\gamma_{HCO_3^-}$ in 10 MM carbon monoxide. Average BHb = 16 MM per liter.

To evaluate P they used Poisson's equation for the variation of the potential about a point having a charge $\pm z\epsilon$. The expression may be briefly written

$$P = \text{funct.}\ (z_A\, \epsilon)$$

from which they obtained

$$h_B = \text{funct.}\ (n_A\, z_A^2\, \epsilon^2) = \text{funct.}\ (z_A^2\, c_A)$$

The assumption of Poisson's equation, that the charges on the ions A act as if at a point, is a sufficiently close one for ions of the

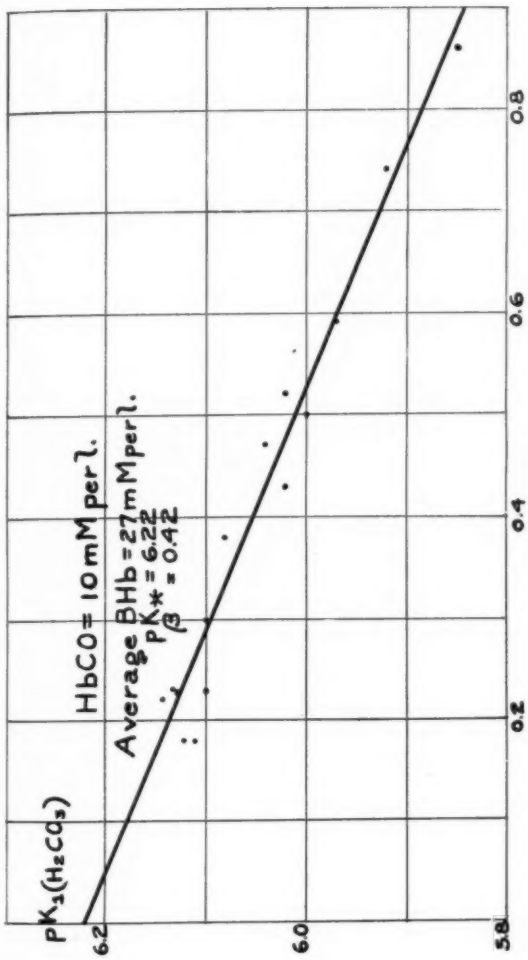

FIG. 2. Log $\gamma_{HCO_3^-}$ in 10 mM carbon monoxide. Average BHb = 26.6 mM per liter.

TABLE I (*Fig. 1*).

Log $\gamma_{HCO_3^-}$ at Varying Γ and Varying BHb.

HbCO = 10 mM per liter.

	BHb	$-\text{Log}\gamma_{HCO_3^-}$	$\sqrt{\Gamma}$ *
	mM per l.		
I	15.2	0.26	0.37
	15.6	0.24	0.30
	16.1	0.18	0.18
	16.4	0.21	0.43
	17.4	0.27	0.37
	17.5	0.31	0.52
Average.....	16.4		
II	22.1	0.31	0.43
	22.9	0.22	0.18
	23.2	0.31	0.37
	24.0	0.20	0.23
	24.5	0.21	0.18
	24.9	0.23	0.23
	25.5	0.23	0.30
	26.7	0.29	0.47
	26.8	0.25	0.38
	27.4	0.46	0.86
	27.7	0.41	0.74
	28.1	0.33	0.50
	28.2	0.36	0.59
	31.1	0.17	0.23
Average.....	26.6		

* Calculated assuming $Hb^{-2\epsilon}$ univalent.

TABLE II.

Log $\gamma_{HCO_3^-}$ at Constant Γ and Varying BHb and $HbCO_3$.

Γ = 133 mM per liter.*

RHb = 10.6 " " "

BHb	$HbHCO_3$	$\text{Log}\gamma_{HCO_3^-}$
mM per l.	*mM per l.*	
20.3	0	0.35
6.7	0	0.41
0	8.2	0.33
0	9.6	0.33
0	13.5	0.37
Average.....		.....0.36 ± 0.026

* Calculated assuming $Hb^{+2\epsilon}$ monovalent.

order of 10^{-8} cm. The colloidal hemoglobin ion has dimensions of an entirely different order of magnitude and we might readily suppose that compared to the ordinary ions the charged points are at infinite distances from each other. Obviously each point would act independently in its influence on B, as if it possessed a

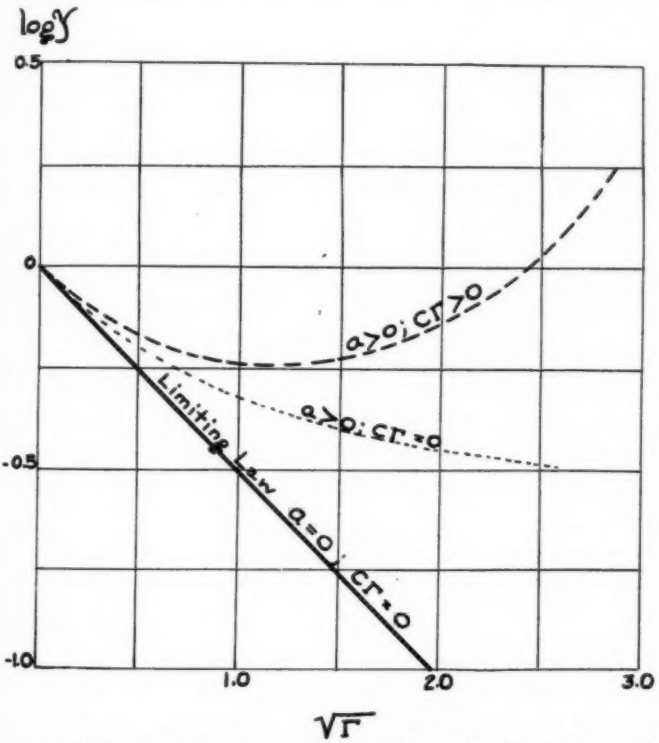

FIG. 3. Theoretical relation of log γ to $\sqrt{\Gamma}$ showing the effects of the three terms of the expanded Debye-Hückel equation.

charge $\pm ze$ where $z = 1$. Whereas a hemoglobin ion may have a stoichiometrical valence of z, its ionic strength valence may be 1 or at least small.

That the charges on the hemoglobin ion act as if at an infinite

distance from each other, or, in other words, that the ionic strength valence of $Hb^{\pm z\epsilon}$ is 1, is indicated by the following experiments. Two series of pK_1' were determined in hemoglobin solution of constant concentration of Hb but varying concentrations of BHb; *i.e.*, $Hb^{\pm z\epsilon}$. The pK_1' values are plotted against $\sqrt{\Gamma}$ (calculated with $z_{Hb} = 1$) in Figs. 1 and 2 from which we have calculated the log $\gamma_{HCO_3^-}$ values given in Tables I and II.

Log $\gamma_{HCO_3^-}$ of the first six points (Table I) with average BHb of 16.4 mM per liter when plotted against $\sqrt{\Gamma}$ may be represented with small deviation by $-\log \gamma_{HCO_3^-} = 0.10 + 0.46\sqrt{\Gamma}$. The remaining fifteen points with an average BHb = 26.6 mM with somewhat more dispersion give $-\log \gamma_{HCO_3^-} = 0.11 + 0.42\sqrt{\Gamma}$. A 60 per cent increase in $Hb^{-z\epsilon}$ produced no appreciable change in log $\gamma_{HCO_3^-}$. Similarly (Table II) even larger changes in the concentration of $Hb^{\pm z\epsilon}$ (positive or negative) produced no change in log $\gamma_{HCO_3^-}$ outside the experimental error.

It is clear than an ionic strength valence of $Hb^{\pm z\epsilon}$ much greater than 1 should have changed Γ sufficiently to alter log $\gamma_{HCO_3^-}$. The absence of this effect is in accord with the assumption that it may be taken as 1.

Ionic Collision Sphere of the Bicarbonate Ion.—Debye and Hückel first considered *all* the ions as charged points. This simplification of Equation 9 gives

$$\log \gamma_i = -\beta_0 \sqrt{\Gamma} \tag{12}$$

the limiting law for very dilute solutions (Fig. 3, heavy line). They next considered the case in which a given ion, B_i, was assumed to be a sphere of finite radius. They state (Debye and Hückel, 1923) that the magnitude a clearly then does not measure the ion radius but represents a length which pictures a mean value for the distance to which the surrounding ions, positive as well as negative, can approach to the given ion. Scatchard (1927) has called a the ionic collision sphere. The value of a, however, should approximate molecular dimensions as determined by totally independent methods and the striking agreement of the theory in this respect is one of its important experimental confirmations. For the common univalent ions a ranges between 2.5 to 5×10^{-8} cm.

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This assumption of an ionic collision sphere of finite diameter makes Equation 9

$$\log \gamma_i = - \frac{\beta_0 \sqrt{\Gamma}}{1 + Ba \sqrt{\Gamma}} \qquad (13)$$

The effect upon the activity coefficient is shown (dotted line) in Fig. 3 in which $- \log \gamma_i$ is plotted against $\sqrt{\Gamma}$. For practically all salts studied (since they have a finite values) the $\log \gamma_i - \sqrt{\Gamma}$ relations are curves of this character. The quite exact adherence (in water) of $\log \gamma_{HCO_3^-}$ against $\sqrt{\Gamma}$ to a straight line with theoretical slope from a concentration $\Gamma = 0.010$ to 0.5 M is unusual. The alternative conclusions to be drawn from this are that a is small or zero or our calculation of $\gamma_{HCO_3^-}$ is erroneous. It should be emphasized that this discrepancy of $\gamma_{HCO_3^-}$ from theory and experience with *mean ion activity* coefficients determined by more rigid thermodynamic methods (*e.g.* in cells without liquid junction) is a serious one and hence the values should be accepted as tentative, especially in the higher concentrations. Without discussing the matter in detail we may point out that the bicarbonate ion is not completely unique in giving $a = 0$ by Equation 9. For example the mean ion activity coefficients, *i.e.* $\gamma_\pm = \sqrt{\gamma_+ \gamma_-}$ of $NaNO_3$ and $NaIO_3$ (determined from the freezing points) and $\gamma_\pm$ of some cobalt amine salts in NaCl (determined by solubilities), follow Equation 10 up to $\Gamma = 0.3$ to 0.5; *i.e.*, the values of a by Equation 9 are zero. The measurements are of course independent of liquid junction potential. Furthermore, Gronwall (1927) has shown that Equation 9 is a special case of a more generalized equation. Briefly Gronwall's conclusion is that the observed 0 or even negative values of a obtained by Equation 9 become finite and positive when calculated by his more general equation. Specifically a value of $a = 0$ by Equation 9 becomes 2.12×10^{-8} cm. We may say then that the adherence of $\log \gamma_{HCO_3^-}$ to Equation 10 is not necessarily contrary to the theory or experiment with other ions.

Dielectric Constant of Aqueous $NaHCO_3$-NaCl Solutions.—Hückel next determined the effect of ions upon the dielectric constant of the solution. He introduced the $G\Gamma$ term into Equation 9, which gives as a complicated function of Γ, the change of

the dielectric constant of the solution with increasing ionic strength. The effect of this dielectric change is to cause log γ_i to pass through a minimum and increase (dash line, Fig. 3). This effect is usually appreciable only at high (1 M) salt concentration. It is absent in the $NaHCO_3$-NaCl solutions studied (up to 0.5 M) as we have seen; hence we shall disregard it completely in the subsequent discussion.

Hydration in Hemoglobin Solutions.—In any aqueous solution of ions or molecules it is necessary to consider the possible occurrence of hydration. We will not discuss the hydration of the ions here except to emphasize Hückel's statement that activity measurements can give no categorical answer as to their existence. On the other hand, a solute like hemoglobin might be considerably hydrated and in a solution of 30 per cent by weight (20 mM per liter) such hydration might have an appreciable effect on the properties of the solution. As in the case of the ions, activity measurements yield no direct answer to the question which must be sought by other methods (optical, chemical).

We can assume either that there is hydration or no hydration; the agreement of the experimental facts with one or the other hypothesis will govern our choice. No hydration of hemoglobin is assumed here since, as we shall subsequently show, the experimental data may be satisfactorily explained on this basis.

Existence of X_{HbCO_3}.—In part we have already discussed the possible occurrence of X_{HbCO_3} in solutions of Na^+, HCO_3^-, CO_2, Hb. We define it no more exactly than to say it is not HCO_3^- ion and have, for simplicity, assumed it = 0. In the main, our data are apparently in accord with this assumption. However, since there is no *conclusive* evidence on this point, if subsequently X_{HbCO_3} can be evaluated our conclusions will have to be modified in part or in whole. As in the case of hydrates, activity measurements cannot determine X_{HbCO_3} or any similar protein ion compound. To call X_{HbCO_3} undissociated hemoglobin bicarbonate is to assume that $NaHCO_3$ is completely dissociated while $HbHCO_3$ is partially dissociated. This is a possible hypothesis, but in view of our conclusion that $HbHCO_3$ behaves as $NaHCO_3$ in this respect we believe it unlikely.

Debye-Hückel Theory in Hemoglobin Solutions.—In our hemoglobin solutions, *i.e.* a mixture of electrolytes and non-electrolytes,

a combination of the Debye-Hückel theory for electrolytes and the principles discussed by Debye and McAulay (1925) and Scatchard (1927) for non-electrolytes gives a satisfactory theoretical approach to our problem. It is necessary to derive a function for the free energy of the bicarbonate ion under all of our experimental conditions in terms of $\gamma_{HCO_3^-}$.

Effect of Hemoglobin on the Free Energy of the Bicarbonate Ion.—Transfer 1 gm.-ion of HCO_3^- at constant concentration and infinite dilution, first, from pure water to a solution of ionic strength = 0, hemoglobin = [Hb], the free energy change = ΔF_*; second, at constant hemoglobin = [Hb] to a solution of ionic strength = Γ. The free energy change = ΔF_0. The total free energy change in both steps is

$$\Delta F_{HCO_3^-} = \Delta F_* + \Delta F_0 \tag{14}$$

We may express these free energy changes as activity coefficients; *i.e.*,

$$\log \gamma_{HCO_3^-} = \log \gamma_* + \log \gamma_0 \tag{15}$$

ΔF_0 is the free energy change at constant dielectric constant and is due to the interionic electrical effects at varying Γ. Log γ_0 is the corresponding activity coefficient, and, as we have already seen, Hückel equated this to

$$\log \gamma_0 = -\beta_0 \sqrt{\Gamma} \tag{16}$$

ΔF_* is the free energy change of transfer from a solution of dielectric constant D_0 to one of dielectric constant D_s when $\Gamma = 0$. Log γ_* is the corresponding activity coefficient.

We will evaluate these free energy terms for hemoglobin solutions and then we can calculate log $\gamma_{HCO_3^-}$ and the corresponding pK_1' at any [Hb] or Γ. We will consider $\Delta F_0 = R\,T \ln \gamma_0$ first.

Dielectric Constant of Hemoglobin Solutions.—In order to calculate log γ_0 we must properly evaluate the β coefficient in hemoglobin, and to do this it is necessary to be aware of the meaning of D_0, the dielectric constant factor contained therein. With rare exceptions solutions of non-electrolytes in water decrease the dielectric constant. The effect of salts except in high con-

centration is small; the enormous electrical forces about an ion, however, (in water a univalent ion at a distance of 3×10^{-8} cm. from its center develops a potential difference of 2×10^{6} volts per cm.) must produce a change (increase) in the dielectric constant in the *immediate neighborhood* of the ion. We have to distinguish, then, three dielectric constants: D_0, that of the pure solvent; D_s, that of the solution as a whole; and D_i, that in the immediate neighborhood of the given ion whose activity is under consideration. The theoretical value of β will vary with our choice of one or the other. On the theoretical grounds Debye and Pauling (1925) excluded D_i from consideration, and in dilute solutions of salts, where D_s is practically constant and equal to D_0, they conclude that the dielectric constant of the pure solvent should be used. The experiments of Brönsted and La Mer (1924) in very dilute solutions, and those reported by Hastings and Sendroy (1925) and ourselves on $\gamma_{HCO_3^-}$ for aqueous solutions of HCO_3^-, are in agreement with this hypothesis.

Non-electrolytes, however, may have as a rule a marked effect on the dielectric constant of water. It is obvious that in Equation 17 $\beta_0 = \dfrac{\text{constant}}{D_0^3}$ must be replaced by $\beta = \dfrac{\text{constant}}{D_s^3}$ where D_s is the dielectric constant of the hemoglobin solution. We will assume that the dielectric constant D_s of a solution of hemoglobin is *increased* proportionately to the hemoglobin concentration and that the increase is different for each derivative of hemoglobin.

On the basis of this assumption alone we will show that the Debye-Hückel theory will account for the behavior of the HCO_3^- ion in our systems and that the two major effects of hemoglobin, *viz.* (1) the decrease of γ_* at $\Gamma = 0$ and increasing hemoglobin and (2) the decrease of β with increase of hemoglobin, are in harmony with the assumption.

Calculation of β at Varying Hemoglobin Concentrations.—Represent the dielectric constant of a solution of any given hemoglobin derivative by an interpolation formula

$$D_s = D_0 (1 + \delta[\text{Hb}] + \delta'[\text{Hb}]^2 + \dots)$$

δ and δ' are constants characteristic of each hemoglobin derivative and give the per cent increase of the dielectric constant per mM of Hb. Experience with other non-electrolytes indicates that we

may neglect higher powers of [Hb] over a short range of concentration; *i.e.*,

$$D_s = D_0 (1 + \delta[\text{Hb}]) \tag{17}$$

Introducing D_s into β we get

$$\beta = \frac{1.86 \times 10^6}{D_s^{\frac{3}{2}} T^{\frac{3}{2}}} = \frac{1.86 \times 10^6}{D_0^{\frac{3}{2}} T^{\frac{3}{2}}} (1 + \delta[\text{Hb}])^{-\frac{3}{2}}$$

Expanding $(1 + \delta\ [\text{Hb}])^{-\frac{3}{2}}$, and neglecting higher powers of δ [Hb], which is subsequently shown to be always less than 0.4, we get as a sufficient approximation, $\beta = \beta_0 (1 - \frac{3}{2} \delta\ [\text{Hb}])$.

Since

$$\beta_0 = 0.54$$

$$\beta = \beta_0 - 0.81\ \delta[\text{Hb}] \tag{18}$$

The qualitative significance of Equation 18 is clear: In hemoglobin solutions β (*i.e.* the slopes of the $\log \gamma_0 - \sqrt{\Gamma}$ lines) should

TABLE III.

Dielectric Coefficient of Hemoglobin Derivatives.

	σ	δ
HbCO	0.0115	0.0142
RHb	0.0214	0.0263
MtHb	0.0260	0.0320
HbCN	0.0160	0.0197
HbNO	0.0160	0.0197

decrease from $\beta_0 = 0.54$, the theoretical value in water. Further, the plot β against [Hb] should give a straight line and if δ varies in different derivatives the β-Hb lines should be different. This agrees exactly with our experimental results given in Fig. 13 of the previous paper and Equation 5; *viz.*, $\beta = \beta_0 - \sigma$ [Hb].

From Equations 5 and 18

$$\delta = 1.23\ \sigma \tag{19}$$

The characteristic experimental constant σ appears on the basis of the theory to be the characteristic constant 0.81 δ giving the

specific effect of each hemoglobin derivative on the dielectric constant of water.

From the observed values of σ in the previous paper, we have calculated δ for the various pigments (Table III). For [Hb] = 15 mM per liter, the calculated dielectric constants at 38° are:

	Calculated D_s.
HbCO	88
RHb	101
MtHb	107
HbCN	94
HbNO	94

To test these calculations we need data on the dielectric constants of hemoglobin solutions directly determined. Complete data of this character are not at hand. Fürth (1923) determined directly by the method of Drude (1897) the dielectric constant of some half dozen biological protein-containing fluids and found that only whole blood, dried red blood cells, and serum gave *higher* dielectric constants than water. In view of the difficulty of the direct determination of dielectric constants of conducting solutions, we can only regard this evidence as suggestive. It is well to emphasize that if hemoglobin *decreases* the dielectric constant, as do most non-electrolytes, β must *increase*. Experimentally we have found the contrary to be the case. This, together with the fact that β decreases proportionately to the hemoglobin concentration (Equation 5), gives support to our assumption and we tentatively conclude that the decrease of β in hemoglobin solutions is due to the increase of the dielectric constant. The hemoglobin derivatives are divisible roughly into two groups according as δ is $>$ or $<$ 0.017. The dielectric constants of solutions of the same concentrations should be different. We have no independent evidence on this point.

Calculation of Log $\gamma_{\pm}$.—Let

F_I = free energy of HCO_3^- ion in the ideal state; *i.e.* in water [Hb] = 0, $c_{HCO_3^-} = 0$, $\Gamma = 0$.

F = free energy at hemoglobin = [Hb], $c_{HCO_3^-} = 0$, $\Gamma = 0$.

The change of free energy in the transfer is given by Debye and McAulay (1925) as

$$F - F_I = \frac{N \epsilon^2 n_{HCO_3^-}}{2D\, b}$$

Differentiating with respect to $n_{HCO_3^-}$ and D and integrating between the limits $n_{HCO_3^-} = 0$ to 1 and $D = D_0$ to D_s, we get as the change in free energy in the transfer of 1 gm.-ion

$$\Delta F_* = RT \ln \gamma_* = \frac{N \epsilon^2}{2b} \frac{D_0 - D_s}{D_0 D_s} \tag{20}$$

N = Avogadro's number = 6.07×10^{23}.
ϵ = elementary electronic charge = 4.77×10^{-10}, E.S.U.
D_s = dielectric constant of the solution.
b = ionic radius of HCO_3^-.
R = gas constant.
T = absolute temperature.
$n_{HCO_3^-}$ = No. of HCO_3^- ions per unit volume.

From Equations 21 and 18 we get

$$F_* = RT \ln \gamma_* = -\frac{N \epsilon^2}{2bD_0} \frac{\delta[\text{Hb}]}{1 + \delta[\text{Hb}]} = -\frac{N \epsilon^2 \delta[\text{Hb}]}{2bD_0} \tag{21}$$

by expansion of $(1 + \delta[\text{Hb}])^{-1}$ neglecting higher powers of $\delta[\text{Hb}]$. Substituting numerical constants (in calories), noting that $D_{0_{38°}} = 72.5$, we obtain, in calories per equivalent

$$\Delta F_* = RT \ln \gamma_* = -\frac{2.29 \times 10^{-8}}{b} \delta[\text{Hb}] \tag{22}$$

from which

$$\log \gamma_* = -\frac{1.06 \times 10^{-8}}{b} \delta[\text{Hb}] \tag{23}$$

The qualitative significance of Equation 23 is clear. Log γ_* (or pK_*) plotted against [Hb] should give a straight line having a value of log $\gamma_* = 0$ (or $pK_* = pK_1$) at [Hb] = 0 with decreasing values of log γ_* (or pK_*) as [Hb] increases. The slopes of these lines $= (1.6 \times 10^{-8} \frac{\delta}{b})$ should vary with the value of δ; *i.e.*, with the nature of the hemoglobin derivative. These are precisely the

results observed as shown in Fig. 12 of the previous paper and Equation 7 of this paper; *i.e.*,

$$\log \gamma_* = -\rho[\text{Hb}] \tag{7}$$

From Equations 23 and 7

$$\rho = \frac{N\varepsilon^2\delta}{2 \times 2.3\, D_0\, RT\, b} = \frac{1.6 \times 10^{-8}\,\delta}{b} \tag{24}$$

The characteristic constant ρ appears to be equal to the ratio of two constants: δ, characteristic of the hemoglobin, and b, characteristic of the bicarbonate ion, multiplied by a factor which includes universal constants and D_0, characteristic of the solvent.

TABLE IV.

Ionic Diameter of HCO_3^-.

Solution.	ρ	$b \times 10^8$ cm.
HbCO	0.012	1.9
RHb	0.021	2.0
MtHb	0.025	2.0
HbCN	0.021	1.5
HbNO	0.012	2.6

Calculation of the Ionic Radius of the Bicarbonate Ion.—Debye and McAulay assumed in deriving Equation 20, that the electric charge of an ion is distributed over the surface of a sphere of radius b, which for simplicity they considered equal to a, the radius of the collision sphere. Similar to a it should have molecular dimensions. From Equation 24

$$b = \frac{1.6 \times 10^{-8}\,\delta}{\rho} \tag{25}$$

Using the δ values calculated from the slopes of the pK_1' lines and the ρ values from the previous paper calculated from log γ_*, we get the value for b given in Table IV. Considering the number of simplifying assumptions made, the agreement of values with each other and with the magnitude of ionic diameters in general as measured by totally independent methods is good.

Restatement of Log $\gamma_{HCO_3^-}$ *in General Terms.*—Equations 1 to 8 give in terms of β, ρ, σ, Γ, and [Hb] the variations of pK_1 and log $\gamma_{HCO_3^-}$ from the experimental data of the preceding paper. It

is now possible to restate these relations in terms of δ, [Hb], b, Γ, and universal constants.

$$\log \gamma_{HCO_3^-} = \log \gamma_0 + \log \gamma_* \tag{26}$$

$$\log \gamma_0 = -\beta_0 \left(1 - \tfrac{2}{3}\delta[\mathrm{Hb}]\right)\sqrt{\Gamma} \tag{27}$$

$$\log \gamma_* = -\frac{N\epsilon^2 \delta[\mathrm{Hb}]}{2.3 \times 2\, b\, D_0\, RT} = -\frac{1.6 \times 10^{-8}\, \delta[\mathrm{Hb}]}{b} \tag{28}$$

$$\mathrm{pK_1} = 6.33 + \log \gamma_{HCO_3^-} \tag{29}$$

$b = 2 \times 10^{-8}$ cm. The values of δ are given in Table III.

DISCUSSION.

The demonstration of marked and specific effects of different hemoglobin pigments upon the pK_1' of carbonic acid rests upon purely experimental grounds and reveals an interesting and unsuspected buffer mechanism in the acid-base equilibrium of the blood. The theoretical explanation of this effect by the Debye-Hückel theory, while simple and plausible, rests upon an almost wholly unsupported assumption which can only be tested experimentally by determinations of the dielectric constants of highly conducting solutions. Obviously, while the explanation is satisfactory, it must be accepted at present as a possible but not exclusive one.

SUMMARY AND CONCLUSIONS.

1. The Debye-Hückel theory is discussed with special reference to its application to concentrated solutions of hemoglobin.

2. Theoretical and experimental reasons are given to support the assumption that the colloidal hemoglobin ion has an ionic strength valence of 1.

3. The relations of the ionic collision sphere of HCO_3^-, the dielectric constant of $NaHCO_3$-NaCl solutions, the hydration of hemoglobin, and the possible existence of X_{HbCO_3} to the activity coefficient of the HCO_3^- ion are outlined.

4. It is shown theoretically that $\gamma_{HCO_3^-}$, the activity coefficient of the bicarbonate ion in hemoglobin solutions, may be divided into two activity coefficients, γ_* and γ_0.

5. γ_* measures the effect of hemoglobin on the HCO_3^- ion. It is theoretically a function of the dielectric constant of the

hemoglobin solution, whereas γ_0 measures the effect of salt on the HCO_3^- ion. Equations were derived giving γ_* and γ_0 as functions of two constants; *viz.*, b, characteristic of the HCO_3^- ion, is its ionic diameter, and δ, characteristic of hemoglobin, gives the effect of the hemoglobin on the dielectric constant of water.

6. The values of b and δ calculated from the observed pK_1' of carbonic acid are consistent with measurements by independent methods. Hemoglobin, contrary to most non-electrolytes, appears to increase the dielectric constant of water.

7. On the basis of the theory it is possible to calculate in terms of universal constants and the characteristic constants b and δ, the activity coefficients of HCO_3^- ion (and hence pK_1') in solutions with Hb varying from 0 to 20 mM per liter and salt from 0 to 0.5 M per liter. The activity coefficients so calculated agree within the limits of experimental error with the observed. The equations for the calculations are

$$\log \gamma_0 = \log \gamma_* + \log \gamma_0$$

$$\log \gamma_* = -\frac{N \epsilon^2 \delta[\mathrm{Hb}]}{2.3 \times 2\, b\, D_0\, RT} = -\frac{1.6 \times 10^{-8}\, \delta[\mathrm{Hb}]}{b}$$

$$\log \gamma_0 = -\beta_0 \left(1 - \tfrac{2}{3}\, \delta[\mathrm{Hb}]\right) \sqrt{\Gamma}$$

From these equations $pK_1 = 6.33 + \log \gamma_0$. $b = 2 \times 10^{-8}$ cm. The δ values are given in Table III. On the basis of δ the hemoglobin derivatives are divided into two groups. (1) reduced hemoglobin, methemoglobin, and cyanhemoglobin; (2) carbon monoxide hemoglobin and nitric oxide hemoglobin. The first group should increase the dielectric constant of water more than the second.

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Explanation of Symbols.

a, a_i = activities of molecules or ions; distance of nearest approach.
$\gamma_{HCO_3^-}$, γ_*, γ_0 = activity coefficients of the bicarbonate ion; Equations 4 and 5, Paper IV.
[ ] = concentrations mM per liter.
( ) = concentrations mM per kilo of H_2O.
$[W]$ = kilos of H_2O per liter.
P_{H_2}, P_{CO_2}, *etc.* = partial pressures of H_2, CO_2, *etc.*
$\alpha^0_{CO_2}$, $\alpha^*_{CO_2}$ = solubility coefficients of CO_2 in water and in a given solution.
E, E.M.F. = electromotive force.
L = liquid junction potential.
$\mathbf{F}$ = 1 faraday.
F = free energy.
e_0 = E.M.F. of reference cell; Equation 3, Paper III.
Γ, μ = ionic strength; Equation 10, Paper V.
z_i = valence.
K_1, K_1', K_* = dissociation constants of H_2CO_3; Equations 3 and 4, Paper IV.
ρ, σ = constants; Equations 37 and 38, Paper IV.
δ, a, b = constants; Equations 9, 18, and 21, Paper V.
β_0, β = constants; Equation 14*a*, Paper IV, and Equation 9, Paper V.
D_0, D_s = dielectric constants of water and a given solution.
ϵ = elementary electronic charge.
N = Avogadro's number or gm. equivalents.
Hb = hemoglobin in general.
RHb = reduced hemoglobin.
HbCO = carbon monoxide hemoglobin.
MtHb = methemoglobin.
HbCN = cyanhemoglobin.
HbNO = nitric oxide hemoglobin.
ln = natural logarithm.
log = 10 base logarithm.
R = gas constant.
T = absolute temperature.